University of Alberta

Role of SNARE isoforms in exocytosis of stored mediators from human granulocytes

by

Michael R. Logan ©

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Experimental Medicine

Department of Medicine

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Abstract

It is postulated that the release of stored mediators from eosinophils and neutrophils is a critical element contributing to the pathogenesis of pulmonary diseases such as asthma. Pre-formed mediators in these cells are stored within cytoplasmic storage granules and small secretory vesicles and released by the process of exocytosis. Several stored mediators have been shown to damage airway epithelium, promote airway constriction and activate other inflammatory cells. The intracellular molecules that are essential for regulating mediator secretion from these cells remain poorly understood.

The formation of a SNARE (SNAP receptor) complex during secretory/granule docking at the plasma membrane has been identified as an essential step for exocytosis. The synaptic SNARE complex is comprised of one vesicular (v-) SNARE, VAMP-2 (vesicle associated membrane protein) and two target (t-) SNAREs: syntaxin-1 and SNAP-25 (synaptosome-associated protein of 25 kDa). Botulinum and tetanus toxins impair exocytosis by specifically cleaving SNAREs, and preventing a functional SNARE complex. It is postulated that distinct SNARE isoforms may, in part, regulate the specificity of vesicle/granule trafficking. The research objective of this project was to investigate the expression and functional role of candidate SNARE isoforms in exocytosis of pre-formed mediators from eosinophils and neutrophils.

Our results demonstrated that the v-SNARE, VAMP-2, was expressed on small cytoplasmic vesicles but not large granules in eosinophils or neutrophils. In contrast, tetanus-insensitive VAMP (TI-VAMP) and endobrevin (VAMP-8) were

expressed on large granules, in addition to membrane fractions containing secretory vesicles. Antibody-mediated inhibition of VAMP-2 in permeabilized cells supported the notion that this v-SNARE is primarily involved in vesicle-mediated trafficking. TI-VAMP, but not VAMP-8, antibodies impaired the release of mediators from more than one granule compartment, suggesting that TI-VAMP is a common element in exocytosis. The t-SNAREs, SNAP-23 and syntaxin-4, were expressed on plasma membranes and shown to interact with each other. Antibody-mediated inhibition of syntaxin-4 suppressed the release of all stored mediators examined, indicating a central role for this isoforms in exocytosis from human granulocytes. In contrast to a previous study, our data did not indicate a significant role for the t-SNARE, syntaxin-6 in exocytosis from either eosinophils or neutrophils. These observations suggest that specific SNARE isoforms play distinct roles in mediator release from human granulocytes. SNAREs and/or regulators of their assembly are, therefore, candidates for targeted inhibition which may prove to be a novel and effective anti-inflammatory treatment strategy.

Preface

This thesis was written in paper format according to the guidelines of the Univeristy of Alberta "Thesis Handbook". Each chapter stands alone as a separate document and is written in the style of the "Journal of Allergy and Clinical Immunology".

Acknowledgements

I would like to dedicate this thesis to

my wife, Anne Logan, for her love and support

I have been fortunate to meet many outstanding people during my graduate training at the University of Alberta. I am grateful for their help in my laboratory training, advice and most of all, their friendship. To all the students and staff who helped with this project by donating blood, I thank you for your generosity.

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List of commonly used abbreviations

AG	azurophilic granule (neutrophil)
AP	alkaline phosphatase
β-Hex	β-hexosaminidase
BAL	bronchoalveolar lavage
BoNT	botulinum neurotoxin
BSA	Bovine serum albumin
Cfeg	free extracellular CG
CG	crystalloid granule (eosinophil)
CLSM	Confocal laser scanning microscopy
COPD	chronic obstructive pulmonary disease
CNT	clostridial neurotoxin
DTT	Dithiothreitol
EE	early endosome
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EPO	eosinophil peroxidase
FACS	Fluorescence-activated cell sorter
IFNγ	Interferon-γ
LDH	Lactate dehydrogenase
LTF	lactoferrin
LE	late endosome
LYS	lysosome

MACS	Magnetic activated cell sorter
MBP	major basic protein
MMP-9	matrix metalloproteinase-9 (gelatinase)
MPO	myeloperoxidase
Munc18	mammalian homologue of *unc18*
NEM	N-ethylmaleimide
NSF	N-ethylmaleimide-sensitive factor
PMD	piecemeal degranulation
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RT-PCR	Reverse transcriptase-polymerase chain reaction
SG	secondary (specific) granule (neutrophil)
SLO	streptolysin-*O*
SM	sec1/munc18
SNAP	Soluble NSF attachment protein
SNAP-23/25	Synaptosome-associated protein of molecular weight 23/25 kDa
SNARE	SNAP receptor
TeNT	tetanus neurotoxin
TG	tertiary granules (neutrophil)
TI-VAMP	tetanus-insensitive-VAMP (VAMP-7)
VAMP	Vesicle-associated membrane protein

CHAPTER 1

I. INTRODUCTION

1. Eosinophil and Neutrophil biology

1.1 Eosinophil and Neutrophil Differentiation

Eosinophils and neutrophils (polymorphonuclear leukocytes (PMNs)) are end-differentiated granulocytes which are critical effector cells in innate immunity.[1;2] Eosinophils were named in 1879 by Paul Ehrlich for their high affinity for the negatively charged dye, eosin.[3] Eosinophils and PMNs are derived from $CD34^+$ progenitors in the bone marrow.[2;4] A common myeloid precursor, the colony forming unit granulocyte/erythroid/ macrophage/ megakaryocyte (CFU-GEMM), gives rise to both eosinophils and PMNs. The CFU-GEMM differentiates further to yield two distinct lineages of precursors, the granulocyte/macrophage-colony forming unit (GM-CFU) and the eosinophil/basophil-colony forming unit (Eo/Bo-CFU). Mature PMNs are derived from GM-CFU under the influence of cytokines interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF).[5] Both eosinophils and basophils are derived from the Eo/Bo-CFU. In addition to IL-3 and GM-CSF, IL-5 is an essential differentiation factor for the production of mature eosinophils from Eo/Bo-CFU.[4;6] IL-5-deficient mice are eosinopenic.[7;8] Similarly, a significant reduction in circulating eosinophils

occurs following administration of intravenous anti-IL-5 neutralizing antibodies in humans.[7;9] In contrast, the overexpression of IL-5 causes profound increases in circulating and tissue eosinophils.[10;11] More recent studies have recognized an additional and critical role for an array of chemokines in granulocyte differentiation. CCR and CXCR chemokine receptors and their ligands have been shown to influence cell cycle progression, apoptosis and recruitment of myeloid precursors and/or $CD34^+$ progenitors.[12-15] Thus, chemokines and the expression of chemokine receptors appear to be critical components of eosinophil and PMN differentiation.

Eosinophils and PMNs exhibit similar transitions from undifferentiated myeloblasts to promyelocytes and myelocytes through mitotic divisions in the bone marrow. The later post-mitotic stages of metamyelocytes and band cells are characterized by maturational processes such as granule protein synthesis and nuclear condensation.[1;2;4;16] For the neutrophil lineage, it takes approximately 4-6 days to pass from the mitotic phases to the myelocyte stage and 5-7 days further for mature PMNs to form.[16] Similarly, eosinophil mature following the mitotic stage for about 8 days prior to their release into the circulation.[4] Approximately 60% of all developing myelocytes in the bone marrow are committed to the neutrophil lineage, while mature neutrophils comprise ~60 % of circulating leukocytes.[16] Eosinophils comprise ~3% of bone marrow and usually do not exceed 3% of the circulating leukocyte population in healthy individuals.[1] In atopic (allergic) individuals, the numbers of peripheral blood eosinophils are, often, elevated and correlate with disease severity.[17-19] Recent studies have demonstrated that $CD34^+$ progenitors that express the α-chain of the IL-5 receptor (IL-5Rα) are upregulated in bone marrow [20] and induced sputum [21] from

allergic individuals following exposure to allergen. Similar observations in nasal mucosal explants have supported that IL-5 mediated differentiation of eosinophil progenitors occurs not only within the bone marrow, but at tissue sites of allergic inflammation.[22]

1.2 Mature Eosinophils and PMNs

Mature eosinophils are approximately 8 μm in diameter with a bilobed nucleus, although three or more lobes is not uncommon.[1] Eosinophils are characterized by their unique large crystalloid granules (CG) (also known as specific or secondary granules). These store cationic proteins (discussed in section 1.6) and take up acidic dyes such as eosin. Additional stored eosinophil mediators are localized to small (or primary) granules and secretory vesicles (SV) (Table 1.1 *pages 15-16*, Table 1.3 *page 18*).[1;23] Following their release from the bone marrow, eosinophils circulate for 8-12 hours and then traffic into specific tissues, where they reside for at least one week.[24] In neonates, eosinophils home to the gastrointestinal tract, thymus, spleen, lymph nodes, and endocrine organs (e.g.: uterus, mammary glands).[25-27]

Mature PMNs are approximately 10-12 μm in diameter and have a highly condensed multilobular nucleus with usually 3-5 lobes. The neutral property of their granules is where the lineage name "neutrophil" is derived from.[16] PMNs contain at least 4 distinct granule populations: large azurophilic (or primary) granules (AG), secondary (or specific) granules (SG), tertiary granules (TG) and SV (Table 1.2, *page 17*).[28] Mature PMNs are stored primarily within the bone marrow, but are also found within the lungs, liver and spleen.[29] The neutrophil storage pool is a cellular source

for host defense against infectious organisms, as evidenced by the depletion of the bone marrow pool in lethal bacterial sepsis.[30;31] Within the vasculature, PMNs are found in the circulation or in direct contact with the capillary endothelium, forming a "marginating pool". In particular, the pulmonary vasculature bed contains a large resevoir of PMNs, some of which are marginating.[32;33] Circulating PMNs exhibit a half-life of approximately 7 hours and, unlike eosinophils, do not reside in any specific tissues until they are recruited by chemotactic agents such as infectious organisms (and/or their products) or chemotactic agents.[2]

1.3 Eosinophil recruitment to asthmatic airways

Eosinophil recruitment and role in allergic inflammation

Asthma is a respiratory disease characterized by: (1) reversible airways obstruction , (2) persistent bronchial hyperreactivity, (3) inflammation and (4) airway remodeling (deposition of connective tissue proteins and development of fibrosis).[34;35] The infiltration of large numbers of eosinophils is a characteristic feature of asthma and has been found to correlate with symptom severity and exacerbations.[36-39] In addition, patients that have died of severe asthma demonstrated a marked elevation in mucosal eosinophils and deposition of eosinophil granule proteins in the bronchi.[40;41]

Eosinophil recruitment is mediated by a polarized T-cell response of the Th2 functional subtype induced following allergen exposure in sensitized (allergic) individuals. Several inflammatory mediators have been identified and implicated in the accumulation and activation of eosinophils, including the cytokines IL-1, IL-3, IL-

4, IL-5, IL-13 and GM-CSF and the chemokines RANTES, monocyte chemoattractant protein (MCP)-3, MCP-4, macrophage inflammatory protein (MIP)-1α and eotaxins-1,2 and 3.[24;42-45] IL-3, IL-5 and GM-CSF exert pleiotropic effects on eosinophils, promoting their survival, differentiation and effector functions.[46;47] IL-4 and IL-13 are potent agonists to induce eotaxin-1 expression in smooth muscle and epithelial cells.[48-51] Eotaxin released from these cells, in turn, recruits and can activate eosinophils for cytokine production [51;52], granule protein release (degranulation) [53-55] and generation of superoxide.[54] In addition to their effector function, eosinophils have been implicated in the generation of the Th2 polarized phenotype. For example, the production of IL-13 is markedly reduced in eotaxin/IL-5 double deficient mice, indicating a role for eosinophils.[56] (Figure 1.1, *page 6*).

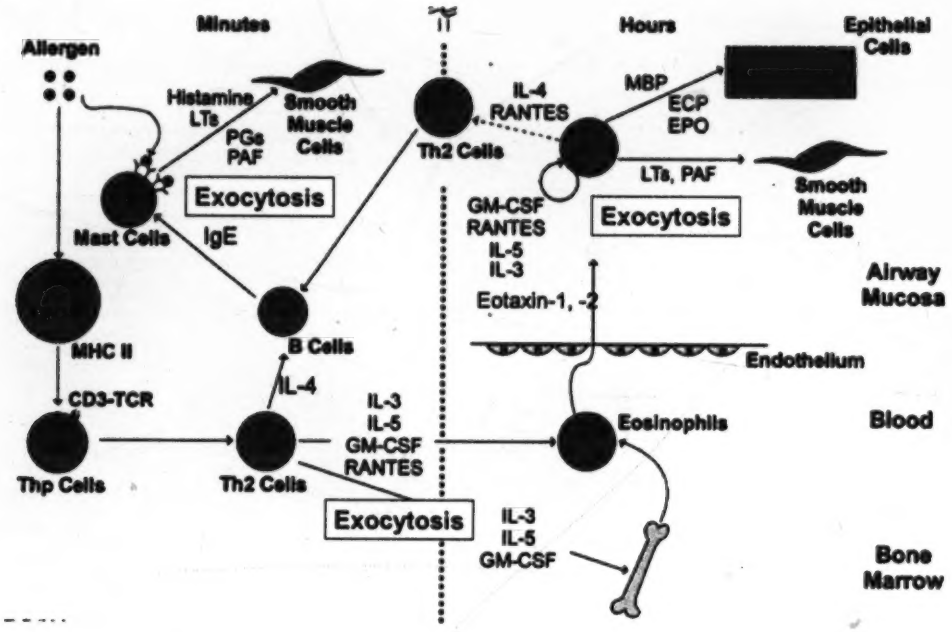

Figure 1.1: Allergen-induced asthmatic inflammation involving recruitment and activation of eosinophils: central role for exocytosis. Following allergen exposure in sensitized individuals, allergen cross-linking of IgE bound to mast cell surfaces triggers rapid degranulation (exocytosis) and the release of mast cell mediators (histamine, prostaglandins (PGs) and platelet activating factor (PAF). Mast cell mediators can activate other inflammatory cells through the release of cytokines and proteases and contribute to pathological features of asthma such as mucus hypersecretion and smooth muscle contraction in small airways (bronchoconstriction). In the chronic day-to-day asthmatic condition a polarized T-cell response of the Th2 subtype predominates, which is characterized by high levels of IL-3, IL-4, IL-5 and GM-CSF. Secreted Th2 cytokines, particularly IL-5, stimulate eosinophilopoeisis in the bone marrow. Th2 cytokines and CC chemokines such as eotaxin-1 and-2 and RANTEs facilitate eosinophil recruitment to sites of allergen exposure. Tissue eosinophils can cause epithelial damage by exocytosis of granule proteins such as major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO). In addition they may produce newly synthesized products such as superoxide, reactive nitrogen species (RNS) and lipid mediators (PGs and leukotrienes (LTs)) which contribute to pathological features of disease such as mucus hypersecretion and bronchoconstriction.
(*Modified from: Moqbel R.1999. Can. Respir. J. 6(5) 453-457*)

Despite the correlation of eosinophil accumulation and symptom severity in asthmatics, it has been difficult to determine a cause and effect relationship due to the lack of eosinophil specific inhibitors. Recently, strategies aimed at specifically impairing eosinophil differentiation and recruitment have focused on targeting IL-5, eotaxins and the chemokine receptor, CCR3.[7;9;57] IL-5-deficient mice are characterized by dramatically reduced bone marrow and blood eosinophil counts as well as absence of eosinophils in the lung following allergen challenge.[8] Deficiency in both IL-5 and eotaxin-1 caused a more pronounced reduction in allergen-induced eosinophila in mice, suggesting that both molecules are critical for coordinating eosinophil recruitment.[56] Studies in patients using humanized anti-IL-5 antibodies have stirred controversy and a strong debate about their efficacy in modulating human asthma.[7] Leckie *et al* reported that blood and sputum eosinophils were significantly reduced in patients that received the anti-IL-5 antibody (Mepolizumab). However, beyond the dramatic drop in the number of blood and sputum eosinophils, there were no changes in lung function parameters when compared to controls. This cast doubt on the pathogenic role of eosinophils in allergic diseases.[9] However, a follow up study reported that while anti-IL-5 treatment significantly depleted BAL and blood eosinophils (>80%), the numbers of bronchial tissue and bone marrow eosinophils were reduced by only 50% and 60%.[58] It has been suggested that this treatment strategy may lack efficacy since it fails to significantly deplete eosinophils within the lung parenchyma.[59]

Eosinophils as immunoregulatory cells

It is important to recognize that eosinophils naturally home to the gastrointestinal tract and other specific tissues but are not found in significant numbers in the lungs of healthy individuals.[25;26;60] Eosinophils within the gastrointestinal tract of healthy subjects exhibit signs of extracellular granule proteins, [60;61] indicating that eosinophil activation and exocytosis are normal processes in the gastrointestinal tract. Similarly, the normal homing of eosinophils to the thymus during development is suggestive of a potential role in T-cell selection.[62] This notion is supported by the observations that eosinophils can act as antigen-presenting cells (APC) to activate T-cells [63;64] and migrate into lymph nodes following administration intratracheally in normal mice [64]. Moreover, IL-5/eotaxin-1 double deficient mice, which are depleted in eosinophils, exhibit defects in T-cell production.[56]

Recent observations from our own laboratory suggest that eosinophils within lymph nodes may play an important role in the polarization of T-cells to the Th2 subtype in allergic subjects. Eosinophils were observed in lymph nodes obtained from asthmatic subjects compared to normal subjects. Peripheral blood eosinophils were found to constitutively express the enzyme indolamine 2, 3 dioxygenase (IDO), which has been previously identified as a critical factor determining apoptosis of T-cells.[65;66] The findings from our laboratory have suggested that eosinophil-derived IDO may contribute to apoptosis of Th1 cells, which may be a mechanism contributing to the Th2 phenotype in asthmatics (Odemuyiwa *et al.* J. Immunol *in press*). Collectively, the above observations indicate that eosinophils may have homeostatic or pathogenic roles, that might may be tissue/site specific.

1.4 Neutrophil recruitment and activation in lung diseases

PMNs are recruited and activated in several respiratory illnesses such as chronic obstructive disease (COPD) [67;68], acute lung injury (ALI) [69;70], acute respiratory distress syndrome (ARDS) [71;72], and cystic fibrosis (CF).[73] Neutrophilia has also been reported in subgroups of asthmatic patients and is frequently observed in cases of severe of asthma, including asthma deaths.[74-76] It is postulated that much of the tissue damage in diseases such as COPD is due to neutrophil-mediated destruction of lung elastic tissue by neutrophil proteinases (elastase, cathepsin G, proteinase 3 and MMPs) in addition to a number of other neutrophil mediators.[68;77] Indeed, in patients with COPD, increased PMNs and secreted neutrophil proteinases correlated with chronic cough and accelerated decline in lung function.[77-79] Similarly, a recent study correlated the numbers of sputum PMNs and the TG mediator, matrixmetalloproteinase- 9 (MMP-9) (gelatinase B), with symptom severity in asthmatic subjects following allergen challenge.[80] Experimental animal models of lung injury have also supported PMNs are critical effectors in the pathogenesis of disease. The depletion of PMNs using anti-neutrophil antibodies [81] and a specific inhibitor of neutrophil elastase [82] were shown to reduce lung injury in animal models.

A central role for the CXC chemokine , IL-8, and the cytokines IL-1β and TNF-α in the recruitment of PMNs to the airways has been indicated in pulmonary disease. Each of these mediators is elevated in BAL and sputum from patients with COPD and ARDS.[44;83-86] IL-8 is a potent chemoattractant for neutrophils, and neutralizing anti-IL-8 antibodies reduced neutrophil accumulation and lung injury in

animal models.[87;88] IL-1β can stimulate the production of a variety of chemotactic molecules, including IL-8, [83] and has been demonstrated to correlate with symptom in ARDS.[89] TNF-α also plays a critical role in amplification of the inflammatory response by stimulating the production of several other cytokines.[83;90] PMNs are, themselves, sources of TNF-α and IL-8 and, thereby, can contribute to their own recruitment and accumulation (Figure 1.2, *page 11*).[91;92]

In addition to the above-mentioned mediators, other CXC chemokines (eg: epithelial neutrophil-activating protein-78 (ENA-78), growth-related oncogene (GRO-α and -β, and granulocyte chemotactic peptide-2 (GPC-2)) have been shown to be involved in neutrophil recruitment.[84;93;94] In a similar manner to IL-8, the leukotriene, LTB_4, mediates neutrophilic inflammation [95;96] and has been shown to be elevated in COPD exacerbations.[97] More recently, IL-17 produced from $CD4^+$ T-cells has been implicated in neutrophil recruitment. This effect appears to be due, at least in part, to IL-17-mediated production of IL-8 by bronchial epithelial cells and possibly other cell types.[98;99]

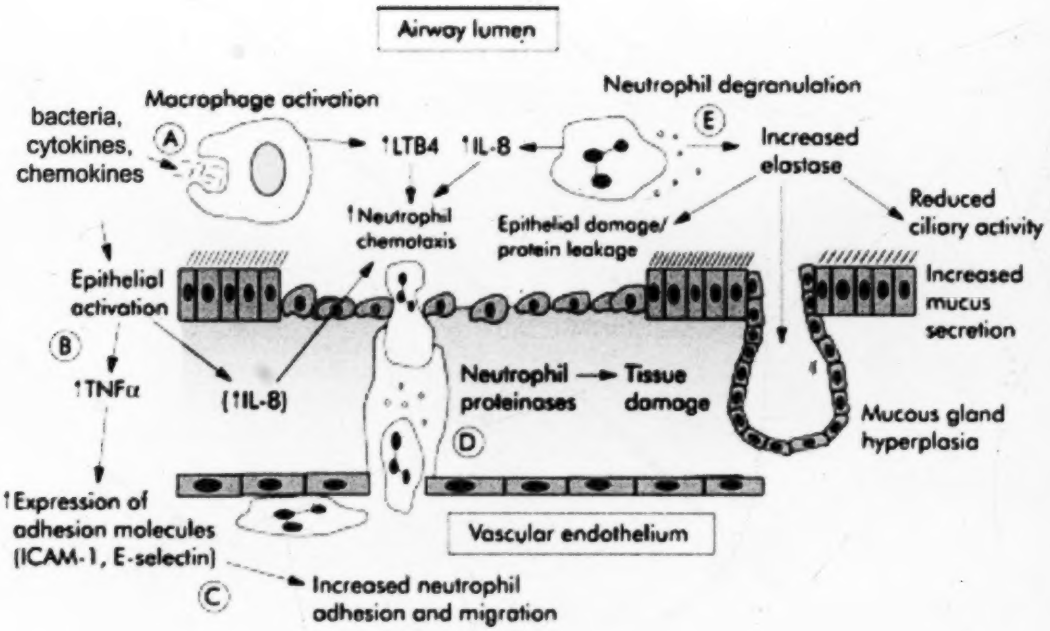

Figure 1.2: Neutrophilic inflammation during exacerbations of COPD.
(A) Macrophage phagocytosis of bacteria and/or activation by chemokines/cytokines (induce release of LTB4 and TNF). (B) Epithelial activation in response to TNF-a and bacterial products (release of TNF-a and IL-8). (C) Activation of endothelium (due to TNF-a (elevated expression of adhesion molecules promoting neutrophil extravasation). (D) Neutrophil migration (follows from binding to adhesion molecules and IL-8/LTB4 chemotactic gradient, elastase release and tissue damage). (E) Neutrophil elastase release in airway (epithelial damage and protein leakage; increased mucus secretion; reduced mucociliary clearance; associated release of IL-8 and LTB4 resulting in amplification of chemotactic signal). LTB4=leukotriene B4; IL-8=interleukin 8; TNF =tumour necrosis factor; ICAM-1=intercellular adhesion molecule 1. *(Modified from: White AJ. Thorax 2003 **58** 73-80)*

1.5 Eosinophil and neutrophil mediators

Eosinophils and PMNs are capable of exerting a wide range of biological effects on tissue and other inflammatory cells through the release of pre-formed mediators localized to granules and SV (discussed in section 1.6) and via *de novo* synthesis and release of products such as lipid mediators and cytokines. Over 29 different cytokines, chemokines and growth factors have been identified in eosinophils, some of which are stored in either CG or SV or both (Table 1.1, *pages 15-16*).[1;23;100] Similarly, a diverse number of pro-inflammatory mediators can be released from activated PMNs (Table 1.2, *page 17*).[92;101]

Like other leukocytes, eosinophils and PMNs contain lipid bodies, which are sites of storage of the enzymes cyclooxygenase and 5-lipoxygenase (5-LO) and their substrate, arachidonic acid (AA). Lipid mediators include: platelet activating factor (PAF), leukotrienes, prostaglandins and lipoxins.[1;102-104] In particular, much attention has been given to the leukotrienes (LTB_4, LTC_4, LTD_4 and LTE_4) for their roles in respiratory diseases.[102;104] Eosinophils have been identified as the principal LTC_4-producing cells in the bronchial mucosa of asthmatic subjects [105] and, in contrast to PMNs, produce little LTB_4.[106] LTC_4 and its extracellular derivatives, LTD_4 and LTE_4, are critical mediators in allergic inflammation in their capacity to induce bronchoconstriction, mucus hypersecretion, and increased vascular permeability.[104;107] In addition, lipid mediators such as LTC_4 and PAF can act in an autocrine fashion to induce exocytosis of eosinophil-derived mediators and the generation of superoxide (O_2^-).[108;109] LTB_4 is a potent chemoattractant and activator for PMNs, and, thus, can contribute in an autocrine fashion to PMN recruitment.[95;96]

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) from granulocytes have been implicated in the pathogenesis of asthma and other inflammatory diseases.[110-112] ROS can directly injure airway tissues.[113] Similarly, the inappropriate formation of nitric oxide (NO)-derived oxidants can damage tissues and can also lead to the modification of protein function through the nitrosylation of tyrosine residues.[114;115] Both eosinophils and PMNs can generate O_2^- by the recruitment and activation of the NADPH oxidase enzyme complex.[116;117] PMNs recruit these components predominantly to the phagosomal membrane for the subsequent elimination of ingested pathogens.[117;118] In contrast, NADPH oxidase components are directed to the plasma membrane in eosinophils, suggesting the extracellular release of O_2^-.[119] Interestingly, eosinophils are capable of generating up to tenfold more O_2^- than PMNs which may be due to their higher expression of protein components of the NADPH oxidase complex.[120]

Superoxide (O_2^-) formed in the phagosome or extracellular space from the activity of the NADPH oxidase is rapidly dismutated by the superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2).[121] In the presence of halides, H_2O_2 is converted to hypohalous acids via the enzymatic activity of eosinophil peroxidase (EPO) and myeloperoxidase (MPO) from eosinophils and PMNs, respectively . EPO has a much higher affinity for Br^- than other halides, leading to the production of hypobromous acid (HOBr). In contrast, MPO preferentially utilizes Cl^- to form hypochlorous acid or bleach (HOCl) (Figure 1.3, *page 19*).[1;118;122;123]. The granule proteins, EPO and eosinophil cationic protein (ECP), have been detected within eosinophil phagosomes [124] indicating that, like PMNs [117;118], granule-phagosome fusion accompanies

phagocytosis. However, PMNs have been shown to have a much greater capacity to eliminate ingested microbes than eosinophils.[125] In addition, EPO and MPO can utilize a number organic and inorganic low molecular weight substances to generate an array of reactive oxidants and diffusible radical species, including nitrating intermediates.[111;112] Interestingly, the generation of nitrogen intermediates by MPO and EPO has been shown to occur in the presence of plasma levels of halides, suggesting this reaction may be favored over the generation of HOCl and HOBr, respectively (Figure 1.3, *page 19*.[111;126]

Table 1.1: Eosinophil cytokines and their intracellular storage sites

Interleukins	mRNA & protein expression	Quantity / 1 x 10^6 cells	Localization
Interleukin-1α	mRNA protein	--	--
Interleukin-2	mRNA protein	6 ± 2 pg	Crystalloid granules (core)
Interleukin-3	mRNA protein	--	--
Interleukin-4	mRNA protein	~75 ± 20 pg	Crystalloid granules (core)
Interleukin-5	mRNA protein	--	Crystalloid granules (core/ matrix?)
Interleukin-6	mRNA protein	25 ± 6 pg	Crystalloid granules (matrix)
Interleukin-9	mRNA protein	--	--
Interleukin-10	mRNA protein	~25 pg	--
Interleukin-11	mRNA	--	--
Interleukin-12	mRNA protein	--	--
Interleukin-13	mRNA protein	--	--
Interleukin-16	mRNA protein	1.6 ± 0.8 ng	--
Leukemia inhibitory factor (LIF)	mRNA Protein	--	--
Interferons and Others			
Interferon-γ(IFNγ)	mRNA protein	--	--
Tumor Necrosis Factor (TNF)	mRNA protein	--	Crystalloid granules (matrix)
Granulocyte/ Macrophage Colony-Stimulating Factor (GM-CSF)	mRNA protein	15.1 ± 0.3 pg	Crystalloid granules (core)
Chemokines			
Eotaxin	mRNA protein	19 ± 4 pg	Crystalloid granules
Interleukin-8	mRNA protein	140 pg	Cytoplasmic
Macrophage Inflammatory Protein-1α (MIP-1α)	mRNA protein	-	-

Modified from: Lacy P; Becker AB; Moqbel R. 2004. In: The Human Eosinophil, Wintrobe's Clinical Hematology 11th Ed. pp. 311-333. Lippincott Williams and Wilkins. Philadephia

Table 1.1 (cont'd)
Eosinophil cytokines and their intracellular storage sites

Chemokines	mRNA & protein expression	Quantity / 1 x 10^6 cells	Localization
Monocyte chemoattractant protein-1 (MCP-1)	Protein	--	--
MCP-3	mRNA	--	--
MCP-4	mRNA	--	--
RANTES	mRNA protein	72 ± 15 pg	Crystalloid granules (matrix) and small secretory vesicles
Growth Factors			
Heparin-Binding Epidermal Growth Factor-Like Binding Protein (HB-EGF-LBP)	mRNA	-	-
Nerve Growth Factor (NGF)	mRNA protein	4 ± 2 pg	-
Platelet-Derived Growth Factor, B chain (PDGF-B)	mRNA	-	-
Stem Cell Factor (SCF)	mRNA protein	-	Membrane, cytoplasm
Transforming Growth Factor-α (TGFα)	mRNA protein	22 ± 6 pg	Crystalloid granules (matrix) and small secretory vesicles
Transforming Growth Factor-β1 (TGF-β1)	mRNA protein	-	-

Modified from: Lacy P; Becker AB; Moqbel R. 2004. In: The Human Eosinophil, Wintrobe's Clinical Hematology 11th Ed. pp. 311-333. Lippincott Williams and Wilkins. Philadephia

Table 1.2: PMN granule populations and their membrane and lumenal proteins

Azurophilic granules (AG)	Specific granules (SG)	Tertiary granules (TG)	Secretory vesicles (SV)
Membrane			
CD63	CD11b/CD18	CD11b/CD18	**Alkaline phosphatase**
CD68	CD15	Cytochrome b_{558}	CD10
Presenelin 1	CD66	Diacylglycerol deacetylating	CD11b/CD18
Stomatin	**CD66b** (formerly CD67)	Enzyme	CD13
Vacuolar H^+ ATPase	Cytochrome b_{558}	fMLP-R	CD14
fMLP-R	NRAMP-1	**CD66b**	
Gα protein subunit	SCAMP	CD16	
Laminin-R	uPA-R	CD45	
Leukolysin	Vacuolar H^+ ATPase	CR1	
NB1 antigen		C1q-R	
Rap-1, -2		Decay-accelerating factor (DAF)	
SCAMP		Leukolysin	
Stomatin		Vacuolar H^+ ATPase	
Thrombospondin-R			
TNF-R			
uPA-R			
Vitronectin-R			
Matrix proteins			
Acid glycerophosphatase	β2-microglobulin		Plasma proteins
Acid mucopolysaccharide	Collagenase	Acetyltransferase	
α1-antitrypsin	CRISP-3 (SGP-28)	β2-microglobulin	
α-mannosidase	Gelatinase	CRISP-3	
Azurocidin	hCAP-18	Gelatinase	
BPI	Histaminase	Lysozyme	
β-glycerophosphatase	Heparanase		
β-glucoronidase	**Lactoferrin**		
Cathepsins	Lysozyme		
Defensins	NGAL		
Elastase	uPA		
Lyzozyme	Sialidase		
Myeloperoxidase (MPO)	Transcobalamin-1		
N-acetyl β-glucosaminidase (β-HEX)			
Proteinase-3			
Sialidase			
Ubiquitin-protein			

Granule membrane and lumenal marker proteins are **bolded**

Modified from: Faurschou,M. & Borregaard,N. 2003. Microbes. Infect. 5(14) 1317-27.

Table 1.3: Granule populations and stored mediators in immature and mature human eosinophils.

Crystalloid (secondary/specific) granules	Primary granules	Small granules	Secretory vesicles
Core			
Catalase			
Cathepsin D			
Enoyl-CoA-hydrolase			
β-Glucuronidase			
Major basic protein			
Matrix			
Acid phosphatase	Charcot-Leyden crystal protein (galectin-10)	Acid phosphatase	Plasma proteins [Albumin]
Acyl-CoA oxidase		Arylsulphatase B (active)	
Arylsulphatase B (inactive)		Catalase	
Acid β-glycerophosphatase		Elastase	
Bactericidal/permeability-increasing protein		Eosinophil cationic protein	
Catalase Cathepsin D Collagenase Elastase			
Enoyl-CoA-hydrolase (also in core)			
Eosinophil cationic protein **(ECP)**			
Eosinophil-derived neurotoxin **(EDN)**			
Eosinophil peroxidase **(EPO)** Flavin adenine dinucleotide (FAD) β-Glucuronidase			**EPO**
β-Hexosaminidase			
3-Ketoacyl-CoA thiolase			
Lysozyme			
Major basic protein			
Phospholipase A_2 (Type II) Nonspecific esterases			
Membrane			
CD63			Cytochrome b_{558} [p22*phox*]
V-type H^+-ATPase			VAMP-2

Modified from: Lacy P; Becker AB; Moqbel R. 2004. In: The Human Eosinophil, Wintrobe's Clinical Hematology 11th Ed. pp. 311-333. Lippincott Williams and Wilkins. Philadephia

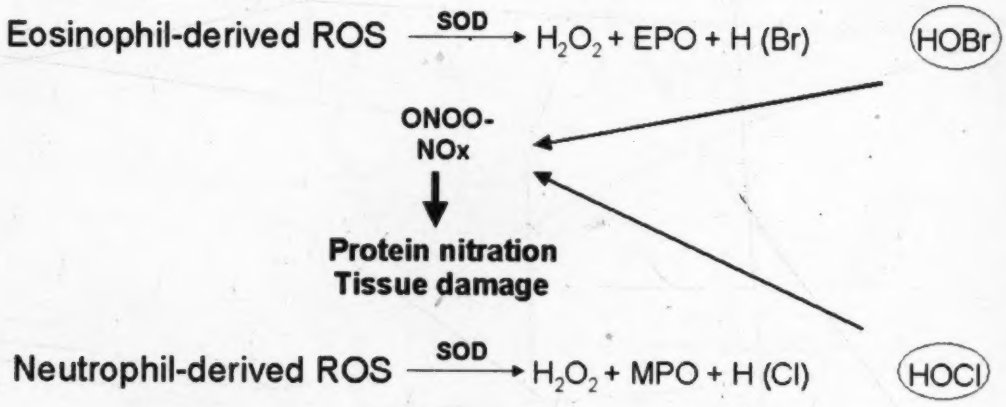

Figure 1. 3
Generation of reactive oxygen and reactive nitrogen species by eosinophil peroxidase (EPO) and myeloperoxidase (MPO).
Superoxide (O_2^-) generated from eosinophils or polymorphonuclear cells (PMNs) is rapidly dismutated by superoxide dismutase (SOD) to generate hydrogen peroxide (H_2O_2). In the presence of halides, (Br^- for eosinophils; Cl^- for PMNs), the enzymes EPO and MPO produce hypohalous acids leading to bromination or chlorination for eosinophils and PMNs, respectively. Alternatively, EPO and MPO can catalyze the formation of a number of reactive nitrogen species (NOx) that are involved in protein nitration. O_2^- produced from eosinophils and neutrophils may also combine with NO produced from epithelial cells and/or inflammatory cells to produce peroxynitrite (ONOO-). ONOO- is microbicidal and can cause nitration of proteins leading to modification of their function.

1.6 Granule populations in eosinophils and PMNs and stored mediators

Eosinophil granules and granule proteins

Human eosinophils contain at least four different populations of membrane-bound granules which store pre-formed mediators: *crystalloid* granules (CG), *primary* granules, *small* granules and *SV* (Table 1.1 *pages 15-16*, Table 1.3, *page 18*).[1]

Crystalloid granules (CG). These are unique large granules (0.5-0.8 μm diameter), also known as *specific* or *secondary* granules, which have a characteristic electron-dense core and an electron-lucent matrix. Approximately 200 CG are found within each mature eosinophil.[1] In mature cells, cationic granule proteins localized to the CG matrix include eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP). In contrast, major basic protein (MBP) is localized to CG cores.[1] The recruitment of eosinophils is a critical component for host defense to parasites, and each of the four cationic proteins is cytotoxic to helminths.[127-129] In addition, CG contain stored cytokines some of which are localized to the crystalline core (eg: IL-2, IL-5, IL-4 and GM-CSF) while others to the matrix compartment (eg: RANTES, IL-6, TNF-α and TGF-α).[1] CG also contain acid hydrolases, similar to lysosomes, and bactericidal proteins (Table 1.3, *page 18*).[1;100]

MBP, named after its abundance (as much as 250 pg per cell), is synthesized during early eosinophiliopoiesis and granulogenesis as a proform (proMBP). ProMBP is found in non-condensed granules in maturing eosinophils, whereas MBP is localized to the electron dense core of CG.[130] It is postulated that post-translational

modifications of proMBP occur within the granule to ultimately form the functional protein. This processing event is necessary for the bioactivity of MBP, as proMBP lacks the cytotoxic and stimulatory effects of MBP.[131] MBP is directly cytotoxic to airway tissues, including bronchial epithelium and pneumocytes[132], and is an effective stimulus for neutrophil superoxide (O_2^-) production and secretion of IL-8.[133;134] MBP has been suggested to contribute to airway hypperreactivity (AHR) in asthma functioning as an allosteric inhibitor of M2 muscarinic receptors on airway nerves.[135]

The basic CG matrix proteins, EPO, ECP and EDN exert a number of effector functions. As described in section 1.5, EPO is an important enzyme involved in the production of reactive oxidants and nitrating intermediates which can cause tissue damage and/or protein modifications (Figure 1.3, *page 19*).[111;112] ECP and EDN are both members of the RNase (ribonuclease) A family, and, unlike EPO, are not exclusively localized to eosinophils. Human PMNs have recently been shown to contain granule stores of EDN and ECP, albeit at much lower levels than eosinophils.[136;137] EDN has ~100 fold higher RNAse activity than ECP and, accordingly, has been shown to be a more effective anti-viral agent.[138] Both ECP and EDN are cytotoxic to nerves and cause hind limb paralysis (Gordon phenomenon) following intraventricular injection in rabbits.[139] The mechanism of ECP-mediated cytotoxicity is unclear, but appears to be independent of its RNAse activity and may involve the formation of channels or pores in the target membrane.[140] ECP and EDN are also implicated in the activation of other leukocytes. ECP, and to a lesser extent MBP, can promote degranulation of mast cells.[141] A selective chemotactic activity of

EDN has recently been reported on dendritic cells *in vitro* and *in vivo*. EDN-mediated chemotaxis was pertussis toxin (PTX)-sensitive and was inhibited by preincubation with a placental RNase inhibitor.[142]

Primary granules. These coreless granules are less numerous than CG, are enriched in Charcot-Leyden crystal (CLC) protein and found in both mature and immature eosinophils. CLC shares significant structural homology with the galectin family of carbohydrate-binding proteins and has been termed *galectin*-10.[143] CLC is abundant in eosinophils (~10% of total cellular protein) and is secreted at tissue sites in eosinophilic disorders.[1]

Small granules. Similar to primary granules, these granules are coreless. Proteins stored within small granules include enzymes: acid phosphatase, arylsulfatase B, catalase and the NADPH oxidase components, and cytochrome$_{b558}$.[1]

Secretory vesicles. These are highly numerous cytoplasmic vesicles, also known as *microgranules* or *tubulovesicular structures* which contain albumin, suggesting an endocytic origin.[1] SV have been reported to contain preformed mediators such as EPO,[144;145] RANTES,[146;147] IL-4 [52;148] and TGF-α [149] The mobilization of SV is postulated to be involved in the rapid and selective release of eosinophil mediators, termed piecemeal degranulation (PMD) (described in section 2.1).

PMN granules and granule proteins

As in eosinophils, all PMN granule populations are enveloped in a phospholipid bilayer and exhibit considerable heterogeneity in their protein contents. PMNs contain at least 4 distinctive granule populations: large *azurophilic* granules

(AG), *secondary* (or *specific*) granules (SG), *tertiary* (or *gelatinase*) granules (TG) and *SV* (Table 1.2, *page 17*).

Azurophilic granules (AG). These are the earliest formed granules in PMNs, also known as *primary* or *peroxidase*-positive granules, and are distinguished by the presence of myeloperoxidase (MPO).[150] As discussed in section 1.5, MPO is a critical enzyme for the generation of reactive oxidants and nitrogen species [112;118;123] (Figure 1.3, *page 19*) which are implicated in protein modification and tissue injury in inflammatory diseases.[110;114;115]. There are two distinct subsets of AG: (i) an early-appearing population which is defensin-poor and (ii) a late-appearing defensin-rich population.[151] Defensins exert cytotoxic effects on a wide variety of bacteria, fungi, enveloped viruses and protozoans by forming membrane spanning pores in the target organism.[152] AG also contain three major serine proteinases, proteinase-3, cathepsin G and elastase. These enzymes are microbicidal, degrade a variety of extracellular matrix proteins (eg: elastin, fibronectin, laminin, type IV collagen and vitronectin) and can induce mediator release from epithelial cells, endothelium and other leukocytes.[150;153] Abnormally high elastase activity is considered to be a major contributing element in the pathogenesis of emphysema, particularly in patients with a deficiency in the plasma protease inhibitor, α1-antitrypsin.[154] Similarly, neutrophil proteinases are implicated in the pathogenesis of COPD.[77-79] It has been proposed by Griffiths and colleagues that since PMN AG and eosinophil CG contain shared enzymes with conventional lysosomes, they be considered "secretory lysosomes" (Table 1.2, *page 17*, Table 1.3 *page 18*).[155]

Secondary (SG) and tertiary (TG) granules

Several antimicrobial proteins are localized to SG and TG (eg: lactoferrin (LTF), human cathelicidin cationic antimicrobial protein-18 (hCAP-18), neutrophil gelatinase-associated lipocalin (NGAL), lysozyme and natural resistance-associated macrophage protein (Nramp)-1). In addition, the membranes of these granules are important sites for intracellular membrane receptors and signaling molecules (Table 1.2, *page 17*, Table 1.3 *page 18*).[150] SG are distinguished by the presence of LTF, a member of the transferrin family of iron-binding proteins. LTF is present in several biological fluids (eg: breast milk and mucosal secretions) and exerts anti-microbial activity by sequestering iron or via the generation of its microbicidal form, *lactoferricin*, following pepsin cleavage.[156] An anti-inflammatory role for LTF has been reported in allergic reactions. LTF is elevated following allergen exposure in sensitized individuals.[157] Recombinant LTF appears to prevent allergen-induced skin inflammation in mice.[158] However, immobilized LTF was recently shown to stimulate O_2^- production and degranulation from eosinophils, suggesting it both pro and anti-inflammatory properties.[159]

TG are enriched in matrix metalloproteinase-9 (MMP-9) (or *gelatinase B*).[160] MMP-9 and other neutrophil metalloproteinases, MMP-8 (collagenase) and MMP-25 (leukolysin) exert a wide range of biological effects through their enzymatic cleavage of extracellular matrix proteins and a variety of other substrates, including chemokines.[161] Excessive metalloproteinase activity is considered to be a major pathogenic factor contributing to tissue remodeling in neutrophilic airway diseases such as COPD and emphysema.[154;162;163]

Secretory vesicles

Similar to eosinophils, PMN SV contain albumin suggesting an endocytic origin.[150] These SV are, however, distinct from a constitutive exocytosis/endocytosis organelle and are not reformed following exocytosis.[164;165]. In PMNs, the population of SV are a rich source of receptors including the β2-integrin CD11b/CD18 (Mac1, CR3), complement receptor-1 CR1, formylmethionyl-leucyl-phenylalanine (fMLP)-receptor, CD14 and FCγIII receptor (CD16). SV are also enriched in alkaline phosphatase and contain plasma proteins and MMP-25.[28;150]

1.7 Eosinophil and PMN granulogenesis

Granule protein synthesis

Although the precise mechanisms regulating granule formation (granulopoiesis) remain to be determined, a variety of studies collectively indicate that distinct granule populations (and stored mediators) arise from the differential expression of granule mediators. In PMNs, MPO is one of the earliest granule proteins to be synthesized and is packaged into AG.[150] At the promyelocyte/myelocyte transition, the production of MPO ceases and defensins are synthesized giving rise to defensin-rich AG.[151] The myelocyte and later band cell stages are characterized by the sequential expression of LTF and gelatinase, giving rise to SG and TG, respectively.[166] In accordance with this "targeting by timing" mechanism of granule biogenesis[150], proteins synthesized during a particular myeloid stage are localized together in the same granules.[151;166] In addition, the overexpression of the SG protein,

NGAL, during the early stage of differentiation in myeloid HL-60 redirects this protein to MPO^+ AG.[167]

Eosinophil granule biogenesis has been less well studied than PMNs. Although a "targeting by timing" model is plausible, further studies are required to determine the precise molecules and mechanisms involved in these processes. The synthesis of eosinophil granule proteins begins at the promyelocyte/myelocyte transition.[168] Eosinophil promyelocytes are abundant in large coreless immature CG (containing MBP, ECP, EDN and EPO)[169] and also contain a few CLC^+ primary granules.[170] In eosinophils derived from $CD34^+$ cells *in vitro*, it was demonstrated that expression of CLC mRNA could be detected following a few days in culture. An overlap, however, in synthesis of granule proteins is suggested, at least for CLC and EPO, as immature granules in these progenitors contained both these proteins by electron microscopy.[170] Similarly, the expression of eosinophil cytokines has been shown to coincide with the stages of granule protein synthesis [171;172]. This may suggest a common pathway for sorting cationic proteins and cytokines to CG during their synthesis. However, cytokines are additionally localized to $albumin^+$ SV[1;100], which are not found in eosinophil progenitors.[173] This indicates a separate mechanism, whereby cytokines and/or growth factors are acquired by eosinophils via endocytosis of plasma mediators.

In the myelocyte and metamyelocyte stages, synthesis of granule proteins begins to decrease and CG condense with the appearance of characterisitic cores.[174] Using patch-clamp capacitance analysis, it has been estimated that exocytosed granules from horse eosinophils and cord blood-derived eosinophils differ in size by

multiples of "unit granules".[175;176] These observations suggest that mature CG arise from homotypic fusions of immature granules which is a similar pattern postulated for granule maturation in neurons and endocrine cells.[177;178] It is unclear if neutrophils follow a similar pattern of unit granule fusion. It is postulated that condensation of CG also involves post-translational processing events for MBP, in particular, and the production of the mature active MBP from proMBP.[131] Such proteolytic events have been described for the neutrophil AG proteins, elastase and cathepsin G, which are similarly synthesized as pro forms, but stored in their active forms.[179]

Once eosinophils reach maturation, MBP is no longer synthesized and messenger RNA (mRNA) is not detectable.[130;180] However, mRNA has been detected for EPO, ECP and EDN in mature cells, indicating that eosinophils have the potential to generate these granule proteins beyond their maturation in the bone marrow.[180] The replenishment of the chemokine RANTES, in particular, following its release from IFN-γ-activated mature eosinophils [146;181] has been reported. This observation indicates that mature eosinophils retain the capacity to synthesize chemokines and/or cytokines, and raises several questions regarding protein sorting mechanisms following bone marrow maturation. It is possible that cytokines synthesized under such conditions may comprise a post-Golgi-derived storage compartment, although no studies have examined this in detail.

Sorting of granule proteins

In PMNs, it is postulated that granule proteins are not sorted by specific target sequences and follow the "targeting by timing" model of granulopoiesis.[150] Although it might be predicted that AG would share similar sorting mechanisms for lysosomes,

significant differences are apparent. MPO is a mannose-6-phosphate (M6P)-labeled protein, similar to other lysosome proteins, but its delivery to AG appears to be independent of the mannose-6-phosphate receptor (M6PR).[182] In addition, other AG proteins such as lysozyme and defensins are not M6P-tagged proteins.[150] AG also do not contain lysosomal-associated membrane protein (LAMP)-1 and –2 [183], which are sorted to lysosomes via a specific targeting sequence in their cytoplasmic tails.[184] However, like eosinophil CG,[185] AG membranes express the tetraspanin, CD63 (LAMP-3),[186] which is localized to late endosomes and lysosomes in other cell types.[187;188] The overlapping distribution of SG/TG and secretory vesicle membrane proteins suggests they may be delivered by a common pathway following their synthesis.[150]

With the exception of a single study, no experimental data are available regarding the sorting of eosinophil granule proteins. Persson *et al* recently reported that LAMP-1 and LAMP-2 were detected on eosinophil CG and cytoplasmic small vesicles that were not albumin positive. Interestingly, binding experiments of M6PR to eosinophil subcellular fractions did not indicate any significant level of M6P-labeled proteins in mature eosinophils.[189] It remains to be determined whether the M6P pathway is important for granulogenesis during the early stages of eosinophil differentiation.

2.0 Exocytosis of stored mediators

2.1 Patterns of exocytosis in eosinophils and PMNs

Eosinophil and PMN granule mediators are released to the extracellular space and/or or the phagosome lumen (for PMNs) by the process of regulated exocytosis. Exocytosis can be divided into distinct sequential steps which include: (i) granule *mobilization*, (ii) *docking*, (iii) *membrane fusion* and (iv) expansion of the *fusion pore*.[190] The molecules and mechanisms regarding these distinct phases of exocytosis are poorly understood in granulocytes. Granule mobilization likely involves the recruitment of signaling molecules (eg: monomeric Rab family GTPases and their effectors) and motor proteins (kinesins and myosins) to the granule membrane and the subsequent migration of granules along microtubules and actin. [191;192] *Docking* collectively refers to the physical interaction of the outer leaflet of the granule with the inner leaflet of the plasma membrane. This occurs through membrane-spanning protein families and additional membrane associated proteins, including SNAREs (see section 2.2). Docking is a prerequisite for the subsequent stages of membrane fusion, pore expansion and granule content release.[190] Whether SNAREs, themselves, are directly involved in the membrane fusion event remains a subject of intense debate (see section 2.3).

The release of granule-derived proteins from PMNs, eosinophils and other granulocytes to the extracellular space has been referred to by many as *degranulation*. *Degranulation*, however, is a complex process in these cell types and at least four distinct modes (or patterns) of stored mediator release have been identified in experimental settings and *in vivo*. *Classical* (or simple) exocytosis occurs by single

granule fusions with the plasma membrane. *Compound* exocytosis is characterized by granule-granule fusions within the cytoplasm followed by a single large exocytotic event resulting from granule membrane fusion with the plasma membrane. *Piecemeal degranulation* (PMD) involves the mobilization and secretion of small SV independent of large granule events and is implicated in the selective release of stored mediators. Finally, deposition of free floating granules and eventually their protein contents may result from *necrosis* or *cytolysis* and the subsequent loss of cell integrity. Mediator release via necrosis, although physiologically relevant, is not considered a form of exocytosis.[1]

Both eosinophils and PMNs are rarely activated while in the circulation, but undergo degranulation following extravasation and migration into tissue sites.[1;2] The examination of tissue and peripheral blood PMNs and eosinophils by several researchers has suggested that the predominant pattern of exocytosis is dependent, at least in part, on the type of the agonist(s) utilized and the cellular microenvironment. As mentioned in section 1.3, it is important to recognize that eosinophil activation and degranulation has been reported in normal healthy tissues.[61] Thus, it must not be misintrepreted that eosinophil degranulation is always associated with disease pathology. In experimental settings, a wide variety of agonists have been shown to activate eosinophils and PMNs. Several of these agonists, if not all, are not selective activators of degranulation as they induce several other cellular responses, including chemotaxis, adhesion, and the generation of various mediators such as leukotrienes and O_2^-. Activators of eosinophils and neutrophils include cytokines [100;185;185;193-195], chemokines and chemotactic factors [52;54;55;196;197], lipid mediators [102;104;198],

complement components [195;199-201], immunoglobulins [202;203] and granule-derived proteins.[133;134;142;159]

Classical and compound exocytosis

Patch-clamp analyses have supported the notion that eosinophils and PMNs do exhibit classical and compound modes of secretion. The sequential secretion of single granules, as indicated by stepwise increases in membrane capacitance, has been observed following intracellular administration of Ca^{2+} and GTPγS into eosinophils from guinea pigs, horses and humans [175;176;204-207] as well as human PMNs.[208;209] Eosinophils stimulated in this manner also exhibit granule-granule fusions characteristic of compound exocytosis.[210-212] Ultrastructural studies have confirmed that compound exocytosis is not limited to experimental manipulation *in vitro*, and eosinophils adhering to helminth parasitic larvae often exhibit this pattern of secretion. For the majority of helminth infections, eosinophil recruitment and attachment to parasites is thought to be critical for parasite containment and clearance.[213;214]

A recent study by Lollike *et al* (2002) indicated that granule-granule fusions occured in PMNs following stimulation with ionomycin. Although a small number of compound events were observed, they accounted for ~20% of the total increased membrane capacitance.[215] It is unclear, however, if PMNs exhibit compound exocytosis at tissue sites.

Piecemeal degranulation (PMD)

PMD was first described by Dvorak and colleagues as a unique pattern of secretion exhibited by tissue basophils in tumor lesions and contact allergy skin lesions. Intact cytoplasmic granules that exhibited content loss were detected, which

were associated with the characteristic presence of numerous small cytoplasmic vesicles.[216] Similar evidence of "eroded" cytoplasmic granules was later identified in tissue mast cells [217] and eosinophils.[218] More recently, it has been shown that PMD is not limited to inflammatory cells. Microscopic analysis has indicated that murine chromaffin cells,[219] and enteroendocrine cells exhibit PMD.[220] PMNs do not appear to release their mediators by PMD, although granule populations are released in a sequential manner following cell activation (described below in: ***Hierarchial granule release from PMNs***).

PMD was first shown in cord blood-derived eosinophils cultured in IL-5.[218] Similar to basophils and mast cells, electron microscopy demonstrated the presence of several cytoplasmic vesicles and partially eroded cytoplasmic CG.[218] Later studies which examined tissues from allergic subjects confirmed that the majority of infiltrating eosinophils exhibit signs of PMD.[221-223] Interestingly, although guinea pig eosinophils exhibit similar PMD,[224] the secretory capacity of murine eosinophils has been questioned in several models of allergic inflammation. Tissue eosinophils, although recruited in abundance following allergen provocation, in murine models have exhibited negligible signs of degranulation.[225;226] However, murine airway lumen eosinophils were recently shown to exhibit signs of PMD following allergen challenge.[227] It is possible that differences in *in vivo* priming and activation of eosinophils may account for phenotypic differences observed between mice and humans. Despite this discrepancy, a pathogenic role for eosinophils has been clearly demonstrated by Lee *et al* (2004) in a murine model of allergic inflammation. A new mutant mouse (PHIL) was generated in this study by a modification of the promoter

region of the EPO gene, which is eosinophil specific. PHIL mice were shown to be completely devoid of eosinophils in the bone marrow, blood and tissue sites, but exhibited normal repetoires of other leukocytes. Allergen challenge in sensitized mice demonstrated that PHIL mice did not develop AHR compared with wild type, providing compelling evidence for a pathogenic role of eosinophils in allergic disease.[228]

It has been suggested that PMD may be a mechanism to permit the gradual release of stored granule mediators over a period of hours or days.[229;230] However, rapid mobilization of SV was reported by our laboratory to coincide with the secretion of the chemokine, RANTES, within minutes following stimulation of human eosinophils with IFN-γ. The secretion of RANTES did not temporally coincide with the mobilization and release of MBP (which remained localized to CG cores), suggesting that SV are involved in the rapid release of some preformed mediators.[146] The release of preformed IL-4 via a vesicle-mediated pathway has, similarly, been reported for eosinophils stimulated with cytokines and CC chemokines.[52;148] The molecules and mechanisms regulating PMD are poorly understood. It was suggested by Dvorak *et al* (1975) that membrane budding of granules may be a mechanism for PMD, generating small vesicles mobilized to the cell periphery following cell activation.[218;230] Alternatively, it has been proposed by Moqbel and colleagues that SV may function as transport carriers for CG-derived mediators during PMD. According to this model, SV are available as a rapidly mobilizable pool for exocytosis of stored mediators and surface membrane proteins. Following their fusion with the plasma membrane, recycled vesicles are postulated to participate in granule-vesicle

fusions and may, thereby, transport CG mediators for secretion in successive rounds of exocytosis (Figure 1.4, *page 35*).[146;231]. Further studies are required to determine if this model occurs in activated eosinophils. To date, electron microscopy and subcellular fractionation analyses of eosinophils have identified vesicles which contained EPO,[144;145] ECP,[232], RANTES [146] and TGF-α.[149] It is presently unclear if cytoplasmic vesicles are homogenous in respect to their stored mediators and membrane proteins. Futhermore, Bandeira-Melo *et al* observed that IL-4, but not RANTES, was released following stimulation of eosinophils with IL-16, RANTES or eotaxin.[148] This observation suggests that it is plausible that there is heterogeneity within the secretory vesicle pool.

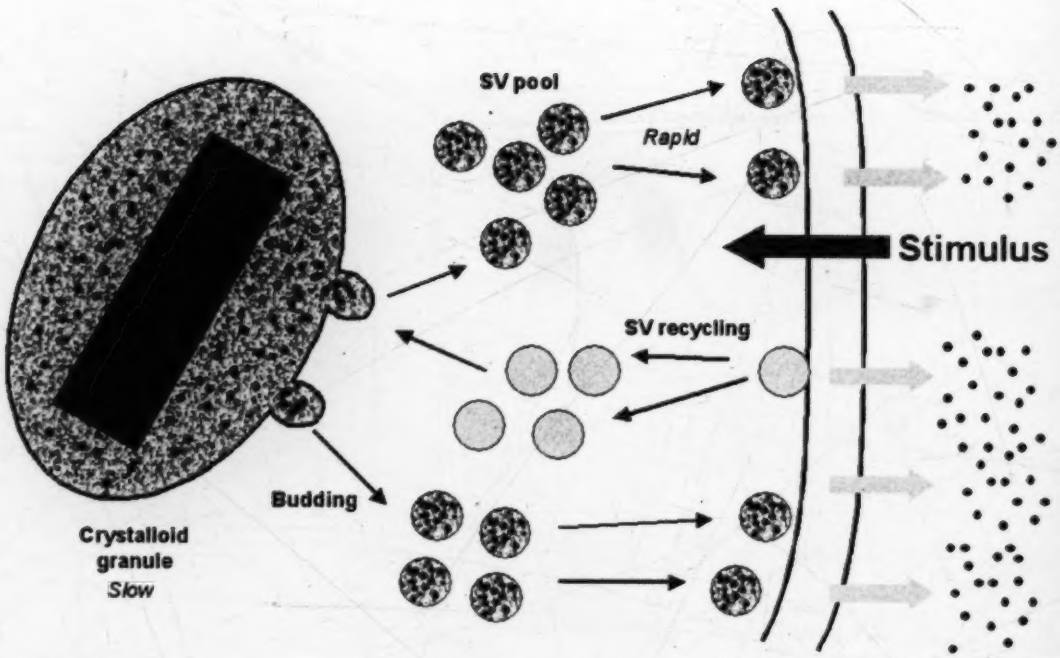

Figure 1.4: Proposed model for eosinophil piecemeal degranulation (PMD). Membrane budding of crystalloid granules may give rise to small vesicles for the slow release of mediators. Alternatively, secretory vesicles are postulated to be mobilized crystalloid granules (CG). Following exocytosis, secretory vesicles are predicted to be recycled to CG to dock and receive intragranular contents. Multiple rounds of vesicle shuttling to the plasma membrane coupled to vesicle recycling is a postulated mechanism for content release of cytoplasmic CG and the resulting formation of intact cytoplasmic CG with eroded matrices and/or cores.

Hierarchial granule release from PMNs

There have been no studies to date that have determined PMD as a mode of secretion for PMNs. However, some similarities to eosinophil PMD are apparent, particularly in the rapid release of SV. Following stimulation with a wide range of agonists, rapid mobilization and secretion of secretory vesicle contents has been observed in PMNs followed by SG and TG exocytosis.[233-236] In contrast, large AG are typically sluggish in their mobilization and, most often, are associated with fusion to the phagosome for elimination of ingested microbes.[118;234;235] Exocytosis of the different granule populations from PMNs can be determined by the examination of secreted marker proteins or the upregulation of granule membrane proteins on the cell surface. The upregulation of surface exposed CD63 and CD66b (formerly CD67) are commonly used markers for examining exocytosis of AG and SG/TG, respectively.[237;238]

The precise mechanisms regulating the sequential release of PMN granules are postulated to involve granule membrane proteins and/or cytoplasmic signaling proteins recruited to the granule surfaces.[150] A critical role for calcium in this process has been indicated, as the hiearchial pattern of release can be mimicked *in vitro* by elevating intracellular Ca^{2+} levels in PMNs.[234;239;240] Recently, Nüsse *et al* demonstrated using patch-clamp analysis that PMN exocytosis exhibited a bi-phasic response following the adminstration of buffered Ca^{2+} solutions. An initial high affinity (low Ca^{2+} : 1.5-5 μM) phase was detected, followed by a low affinity (high Ca^{2+} : ~100 μM) phase. The high and low affinity phases correlated with the release of peroxidase-negative granules (SG and TG) and AG, respectively.[241] This observation

suggests that the duration and intensity of intracellular Ca^{2+} levels may determine the pattern and extent of degranulation.

Necrosis (cytolysis) as a mode for cell free granule-protein deposition

Granule-derived proteins may be released into the extracellular space if the integrity of the plasma membrane is compromised and subsequent death occurs by necrosis (or cytolysis). The examination of human tissue from sites of allergic inflammation has suggested that relatively few tissue eosinophils undergo apoptosis.[221;222;242;243] However, cytolysis of tissue eosinophils has been supported by the observation of cell-free eosinophil CG in the extracellular space (Cfegs) by electron microscopy. The resulting extensive release of granule proteins is considered to be a significant factor contributing to disease pathology in allergic diseases.[221;222;224] Similarly, the balance between the clearance of apoptotic PMNs and the accumulation of necrotic PMNs is a critical component contributing to the severity of neutrophilic diseases such as COPD.[67;68;244]

2.2 SNAREs and the SNARE complex

SNAP receptors (SNAREs) are a highly conserved family of small (10-35 kDa) membrane-associated proteins which were first identified in bovine brain by Rothman and colleagues for their ability to interact with two cytosolic proteins, N-ethylmaleimide-sensitive factor (NSF) and soluble-NSF attachment protein (SNAP).[245] SNAP and NSF had been previously shown to be required for Golgi vesicle trafficking in a cell-free assay [246;247] and have since been demonstrated to be critical in several intracellular trafficking pathways.[248;249] Research efforts by several

groups over the past decade have identified several isoforms of SNAREs and established this family has a key role in not only neurotransmitter release at synapses, but also several other pathways of intracellular membrane trafficking.[250-254]

SNAREs are involved in the distal stage of membrane-bound transport during vesicle/granule docking with the target membrane. The proteins identified by Rothman and colleagues in 1993 [245] had previously been reported to localize to either the vesicle or presynaptic (plasma) membrane in neurons.[255-257] This led to the proposed hypothesis by Sollner *et al* (1993) that targeting of transport vesicles/granules would be mediated by two functionally distinct classes of SNAREs, vesicular (v-) and target membrane (t-)-SNAREs.[245] The synaptic SNARE complex is comprised of the v-SNARE, vesicle-associated membrane protein (VAMP)-2 (previously termed *synaptobrevin*) and its cognate t-SNAREs, synaptosome-associated protein of 25 kDa (SNAP-25) and syntaxin-1 (Figure 1.5, *page 41*). Structural and sequence comparisons between the synaptic SNARE complex and a distantly related SNARE complex [258;259] have indicated a conserved arrangment for SNARE interactions. All SNAREs contain a positionally conserved 60-70 amino acid "SNARE motif" which consists of a repeating heptad of hydrophobic residues. VAMPs and syntaxins, which are both membrane-anchored proteins, contain a single copy of this motif. SNAP-25 and its related isoform, synaptosome-associated protein of 23 kDa (SNAP-23), contain two SNARE motifs and interact with membranes via palmitoylated cysteine residues (Figure 1.6, *page 42*).[260-262] SNARE motifs assemble into parallel four helix bundles [259;263] which are highly stable, being resistant to denaturation by sodium dodecyl sulfate (SDS) [264] and unfold only when either heated to high temperatures (>82 ^{o}C) or

exposed to strong denaturants.[265] Based on these structural analyses, a second nomenclature was proposed that categorized SNAREs in terms of key residues required for assembly: either arginine (R-SNAREs) or glutamine (Q-SNAREs).[266] According to this classification, a SNARE complex is comprised of four helices: one R helix contributed by a VAMP isoform and three Q helices: one from syntaxin (Qa) and two from SNAP-25 (Qb and Qc) (Figure 1.5, *page 41*). While the majority of SNARE complexes appear to follow to this arrangement, exceptions are possible. In particular, homotypic fusion events in the ER are mediated by two Q-SNAREs without a requirement for an R-SNARE. This fusion event is mediated by the NSF-related ATPase, Cdc48p.[267]

As indicated above, the role of SNAREs and SNARE complex assembly in exocytosis has been extensively examined in synaptic vesicle release from neurons. In contrast to several other cell types, a number of morphologically-docked synaptic vesicles are localized to the plasma membrane in the resting state. These synaptic vesicles, termed the readily releasable pool (RRP) are rapidly released in a Ca^{2+}-dependent manner following the generation of an action potential. Following their release, a second more numerous pool of vesicles (the reserve pool) is recruited to the plasma membrane for future rounds of exocytosis. The number of vesicles within the RRP is an important component in determining the extent of neurotransmitter release following stimulation. In addition, the rate of replenishment of RRP from the reserve pool is a critical factor that determines the responsiveness of the synapse during repetitive stimulation (Figure 1.7, *page 43*).[268;269]

It has been proposed that the formation of SNARE complexes between two membranes (*trans* configuration) may be sufficient to overcome the energy barrier between the two opposing membranes to cause membrane fusion. In support of this, Rothman and colleagues have demonstrated SNAREs, alone, appear to be sufficient to induce membrane fusion and lipid content mixing using a cell-free proteoliposome fusion assay.[270] Recent structural analyses and biochemical studies have similarly supported a model in which "zippering" of SNARE motifs proceeds from the NH_2-terminal ends towards their membrane anchors which may "pull" membranes together.[271-273] According to this model, following membrane fusion, *cis*-SNARE complexes (in the same membrane) are disassembled by SNAP and NSF thus allowing SNAREs to return to their "energized" state for future rounds of exocytosis (Figure 1.8, *page 44*).[253;274;275] It has been suggested that NSF can mediate disassembly of *cis* but not *trans* configured SNARE complexes, thus ensuring the progression of the "zippering" process.[276] A role for NSF in SNARE complex disassembly and recycling is supported by the observation that a temperature-sensitive NSF mutant causes accumulation of SNARE complexes at the plasma membrane and loss of neuronal exocytosis in *Drosophila*.[277]

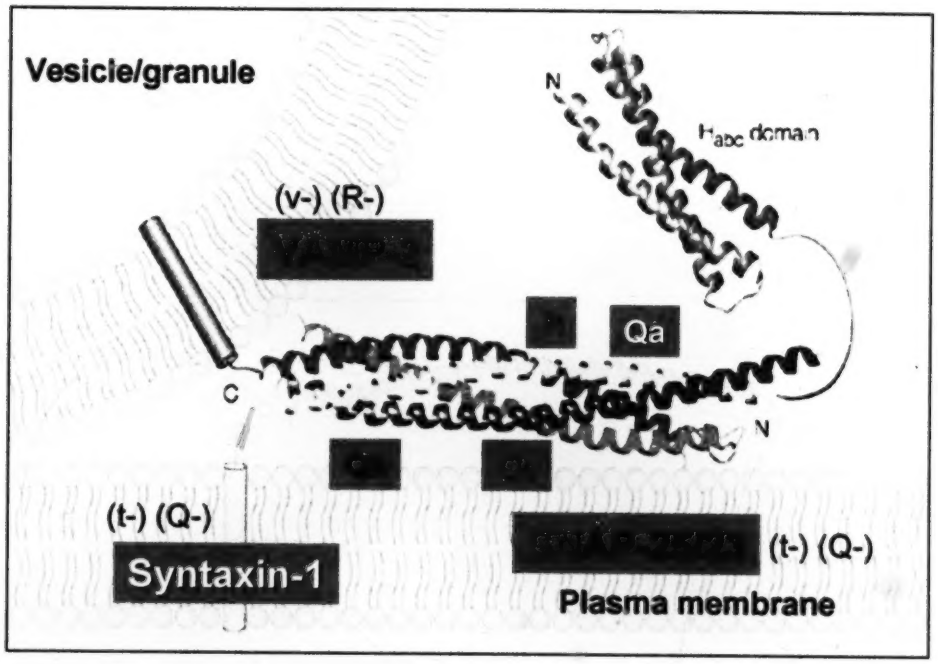

Figure 1.5. Model of the neuronal SNAREs assembled into the core complex.
The ribbon diagrams represent the crystal structure of the core complex and the NMR structure of the amino-terminal Habc domain of syntaxin 1. The amino terminal region of syntaxin 1 (Habc domain) is coloured in orange and the SNARE (SNAP receptor) motifs are colour coded as follows: vesicular (v-) or (R-) SNARE: VAMP-2, red; target (t-) or (Q-) SNAREs: syntaxin 1, yellow, and synaptosomal-associated protein of 25 kDa (SNAP25): amino terminus, blue; SNAP25 carboxyl terminus, green. The cylinders represent the transmembrane regions of synaptobrevin and syntaxin 1, which are inserted into the synaptic vesicle and plasma membranes, respectively. The curved lines represent short sequences that connect the SNARE motifs and the transmembrane regions, as well as the linker region between the Habc domain and the SNARE motif of syntaxin 1. The SNARE complex is a four helix bundle composed of one "R" coil and three "Q" coils: one from syntaxin-1 (Qa) and two from SNAP-25 (Qb and Qc).
(Modified from: *Rizo J. & Sudhof T. Nature Rev. Neurosci. 2002. 3(8) 641-653.)*

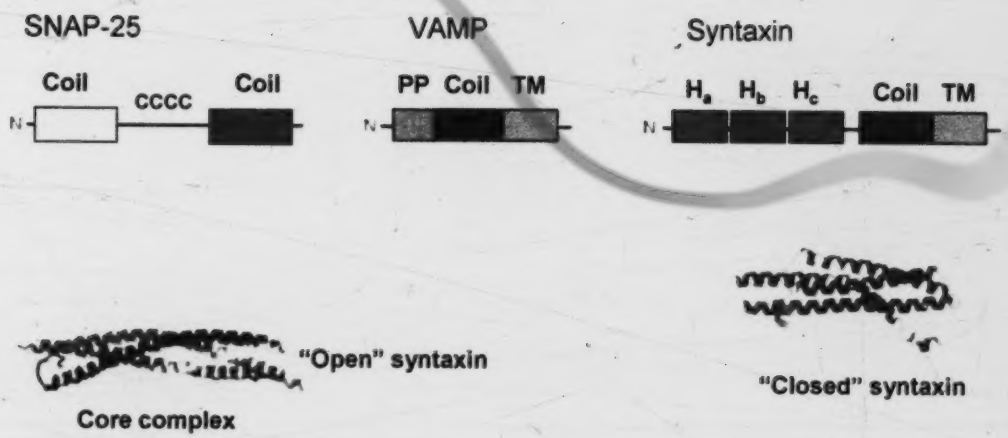

Figure 1.6. SNARE domains. The amino-terminal domain of syntaxin forms a three-helix bundle (red) that binds to its carboxy-terminal coil domain (purple), forming the closed conformation (right), which is bound and stabilized by sec-1/munc-18 (SM) family proteins (described in Chapter 6). A conformational change then occurs to allow dissociation of the SM protein and the opening up of syntaxin, facilitating core complex formation. The coil domains of syntaxin, SNAP-25 and VAMP form the four-helix bundle core complex (left). In addition to the coil domain, VAMP harbours a proline-rich amino-terminal domain (PP) and SNAP-25 harbours a central domain that contains four palmitoylated cysteine residues (CCCC). (SNAP-25, 25 kDa synaptosome-associated protein; TM, transmembrane domain; VAMP, vesicle-associated membrane protein.)
(Modfied from: *Chen YA & Scheller RH. 2001. Nature Rev. Mol. Cell Biol.* **2** 98-106)

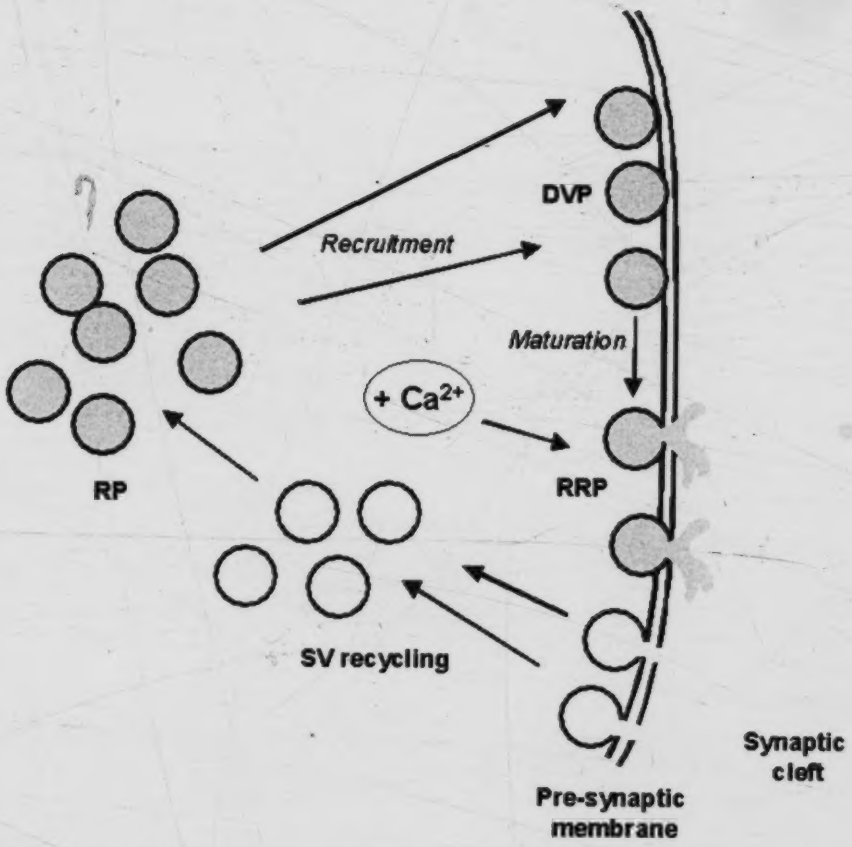

Figure 1.7. Scheme of releasable pools in synaptic vesicle exocytosis. On the basis of different kinetic phases of release and the presumed sequence of events occurring before the actual release of a vesicle, pools of vesicles have been distinguished that differ in their state of release readiness. The *readily releasable pool* (RRP) consists of fully primed vesicles that only await the final calcium trigger for release. This pool is released within some tens of ms (<40 ms for melanotropes, <140 ms for chromaffins) upon a fast rise in internal calcium. The *docked vesicle pool* (DVP) consists of docked vesicles that require maturation steps before release can be triggered by calcium. Release of vesicles from the DVP occurs within 1 s upon a stepwise rise in internal calcium. The *reserve pool* (RP) consists of vesicles that are still linked to the cytoskeleton. These vesicles are only released >1 s after a calcium pulse and their release requires the presence of ATP
(Modified from: ***Kits KS & Mansvelder HD. Brain Res Brain Res Rev. 2000 Aug;33(1):78-94.***)

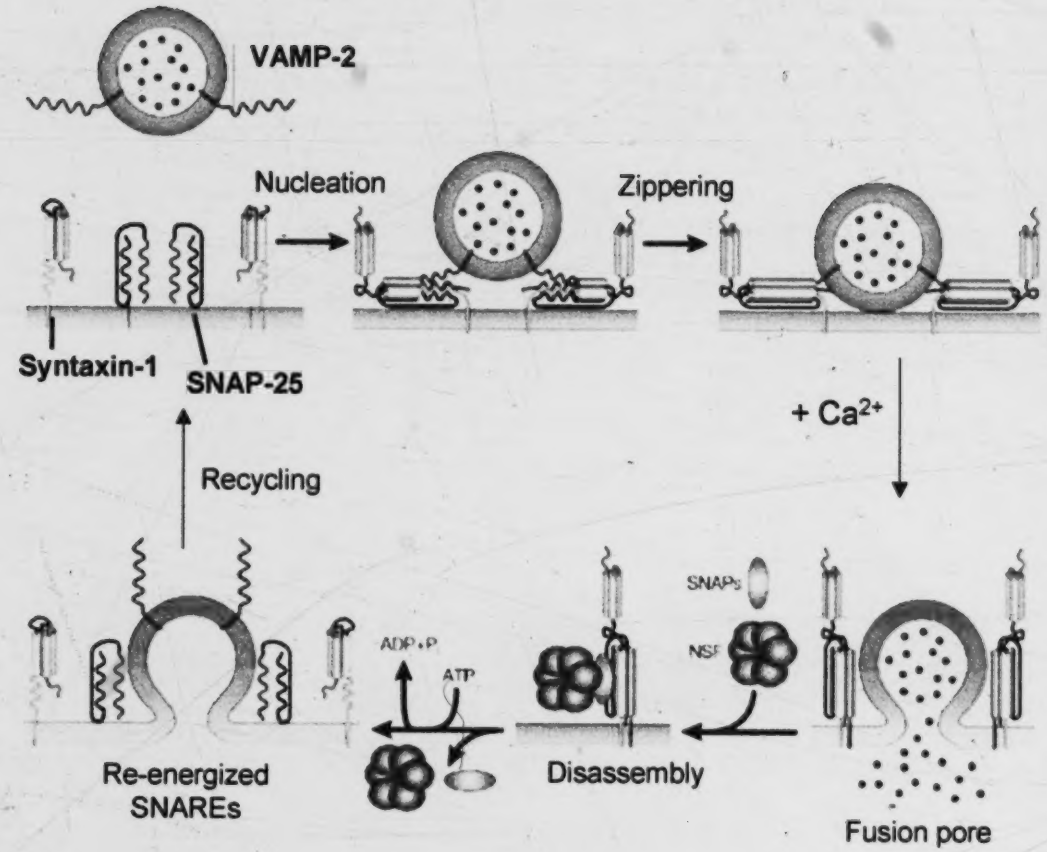

Figure 1.8. Cycle of assembly and disassembly of the SNARE complex in synaptic vesicle exocytosis. Syntaxin exists in a closed conformation that needs to open to initiate core-complex assembly (nucleation). 'Zippering' of the four-helix bundle towards the carboxyl terminus brings the synaptic vesicle and plasma membranes towards each other, which might lead to membrane fusion. After fusion, *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF-attachment proteins (SNAPs) disassemble the *cis*-core complexes that remain on the same membrane to recycle them for another round of fusion (energized state of SNAREs). SNAP25, synaptosomal-associated protein of 25 kDa; SNARE, SNAP receptor.
(Modified from: *Rizo J. & Sudhof T. Nature Rev. Neurosci. 2002. 3(8) 641-43*)

2.3 SNAREs as determinants of trafficking specificity and membrane fusion

Specificity of SNAREs

It was originally proposed by Sollner *et al* (1993) that the specificity of membrane trafficking may be due, at least in part, to the unique and non-overlapping distribution of v- and t-SNAREs. In this simplistic model, only specific (cognate) sets of SNAREs would be anticipated to interact to yield a productive fusion event.[245] The diversity of SNARE isoforms, 20-50 or more distinctively localized SNAREs in eukaryotes from yeast to humans, has indicated that there is certainly a potential for this role. A number of studies have suggested that different intracellular trafficking pathways utilize distinct subsets of SNARE isoforms.[250;252;258;278] Moreover, it has been shown that few non-cognate SNARE pairings induce fusion in cell-free fusion assays.[279;280]

Other studies have indicated that the specificity of SNAREs is not absolute and that additional regulatory molecules are likely required to direct vesicle traffic. Studies of yeast SNAREs have demonstrated that isoforms may participate in more than one trafficking step [281;282] and can compensate for the deletion of a related family member.[283] The normally lethal mutation of SNAP-25 in *Drosophila* has been shown to be prevented by overexpression of SNAP-24, a related isoform.[284] Similarly, it was demonstrated that overexpression of SNAP-23 could rescue the impaired secretion induced by botulinum-E toxin (which specifically impairs SNAP-25: *see section 2.4*) in insulin secreting HIT cells.[285] The interaction of recombinant SNAREs *in vitro* is also promiscuous.[286;287] However, this observation may be due to the loss of correct

protein conformation, as recombinant proteins are not membrane-anchored. A recent study indicated that a region of the SNARE motif of VAMP-2 interacts with its anchoring membrane. This region was shown to be critical for the regulation of complex formation with syntaxin-1 and SNAP-25.[288] Thus, the spatial geometry of SNAREs anchored in their membranes is likely a critical element that determines *trans* complex formation.

Are SNAREs the minimal machinery for membrane fusion?

Despite the universality of SNAREs in membrane trafficking, several observations have cast doubt on whether *trans*-SNARE complexes are the minimal protein machinery required for membrane fusion. Deletion or mutational inactivation of synaptobrevin homologues in *Drosophila* [289], *C.elegans* [290] have demonstrated a severe, but not complete, defect in nerve secretion. Similarly, synapses from VAMP-2-deficient mice exhibited ~100 fold decrease in neurotransmitter release which was correlated with a slow rate of fusion efficiency of the RRP.[291] Interestingly, VAMP-2 deficient mice do not have gross morphological abnormalities in the brain nor significant levels of neurodegeneration, but die shortly after birth due to paralysis.[291] It has been postulated that tetanus-insensitive VAMP (TI-VAMP) (also known as VAMP-7), in particular, may compensate for the loss of VAMP-2 and/or may play a more dominant role in nerve development.[268;292;293] Similar to VAMP-2 knock-outs, neurons from SNAP-25-deficient mice are not impaired in their growth and exhibit a greatly reduced, but not completely abolished, secretory response.[294] In contrast, deletion of syntaxin in *Drosophila* caused a more severe impairment in neurotransmission.[284] It has been postulated that this may be due to the interaction of

syntaxins with additional regulatory molecules such as the sec-1/mammalian homologue of unc-18 (munc-18) family which are essential for nerve development and secretion.[295;296]

Collectively, the observations from SNARE knock out animals suggest that SNAREs may act as fusion catalysts to enhance the rate of membrane fusion. Studies of cortical vesicle exocytosis from sea urchin eggs and yeast vacuole fusion have also provided evidence for different models of exocytosis regarding SNARE function. In contrast to the SNARE zippering model, the disassembly of *cis*-SNARE pairings has been linked to priming events required for homotypic vacuole fusion in yeast.[297;298] A recent study has shown that the ATP-dependent step mediated by SNAP (Sec17p) and NSF (Sec18p) is required to release a soluble SNARE, Vam7p, from the vacuole membrane which is incorporated into and required for *trans*-SNARE assembly. The Sec17p/Sec18p ATP-dependent step can be by-passed by the addition of exogenous soluble Vam7p which permits *trans*-SNARE pairing and subsequent membrane fusion.[299] Exocytosis of morphologically docked cortical vesicles from sea urchin eggs, similar to RRP synaptic vesicles, is similarly elicited in response to Ca^{2+}. However, fusion of cortical vesicles does not appear to coincide with SNARE complex assembly.[300;301] In addition, *trans*-SNARE complexes may be disrupted by excess Sec17p/Sec18p during yeast vacuole fusion without influencing the fusion rate.[302] These observations suggest that SNAREs likely function to hold vesicles/organelles in close proximity to the acceptor membrane, but are not directly involved in the fusion event itself.

2.4 Botulinum and tetanus toxins: research and clinical applications

The identification and classification of SNAREs by Sollner *et al* [245] were paralleled with the discovery that these were also the substrates of the clostridial neurotoxins (CNT), botulinum (BoNT) and tetanus (TeNT).[303-306] CNTs are well characterized for their ability to drastically impair secretory function in neurons and endocrine cells due to specific cleavage of SNARE isoforms.[307-309] In addition, the intracellular administration of CNTs in non-neuronal cell types has been shown to impair vesicle/granule trafficking.[237;310-313] Localized injections of botulinum toxins (serotypes BoNT-A,-B) are beneficial for the clinical treatment of tension headaches and muscle spasticity disorders. In addition, the use of these neurotoxins is being extended for both cosmetic and clinical applications.[314]

CNTs are the products of the *Clostridium botulinum* and *Clostridium tetani* and are synthesized as a single polypeptide chain of ~150 kDa. Processing of the toxin results in a functional di-chain toxin that is comprised of a heavy chain (HC) (~100 kDa) and light chain (LC) (~50 kDa). The heavy chain contains a ganglioside-binding region, which targets the toxin specifically to neurons. Following endocytosis from the receptor surface, the N-terminal portion of the HC facilitates pore formation in the lipid bilayer, injecting the enzymatic LC into the cytosol. The LCs of CNTs function as zinc-binding endoproteases to cleave specific SNARE isoforms (Figure 1.9 *page 50*, Figure 1.10, *page 51*).[308]

There are seven serotypes of botulinum toxins, BoNT-A-G that exhibit differences in substrate specificity for VAMP, syntaxin or SNAP-25 family proteins. The selectivity of BoNTs and TeNT is mediated by their interaction with a specific

binding region on the SNARE protein (confusingly termed the "SNARE-binding motif" which is not synonymous with the motif for SNARE-SNARE interactions). Two copies of the binding motif are present in VAMP-2 (V1 and V2), two in syntaxin1A (X1 and X2) and four in SNAP-25 (S1-4) (Figure 1.11, *page 52*).[309] Ternary SNARE complexes [263] are functionally resistant to CNT cleavage which is thought to be due to masking of either the cleavage site and/or the toxin-binding regions. Thus, the ability of CNTs to impair membrane trafficking depends on the accessibility of SNARE-binding sites.[315] In neurons, toxin accessibility to SNAREs is influenced by activation state and may occur more efficiently once secretion is initiated.[316;317]

Depending on the toxin, assembly of SNARE complexes may either be inhibited or lead to assembled complexes, which are uncoupled from the membrane. For example, BoNT-D, E and F all impair SNARE complex formation. In contrast, the cleavage product of VAMP-2 resulting from BoNT-B, G and TeNT treatment can still form complexes, but is dissociated from the vesicle membrane.[309] Other trafficking steps have been shown to be resistant to TeNT and BoNT treatment. Early observations of TeNT-resistant vesicle trafficking in polarized cells [318] and developing neurons [319] were postulated to involve a SNARE-independent mechanism. However, these trafficking steps have been shown to be mediated by a tetanus-insensitive VAMP (TI-VAMP) isoform.[292;320;321] Similarly, other SNARE-mediated trafficking steps may either be resistant or sensitive to these toxins due to sequence variations within the CNT cleavage sites of the SNARE isoforms.[309]

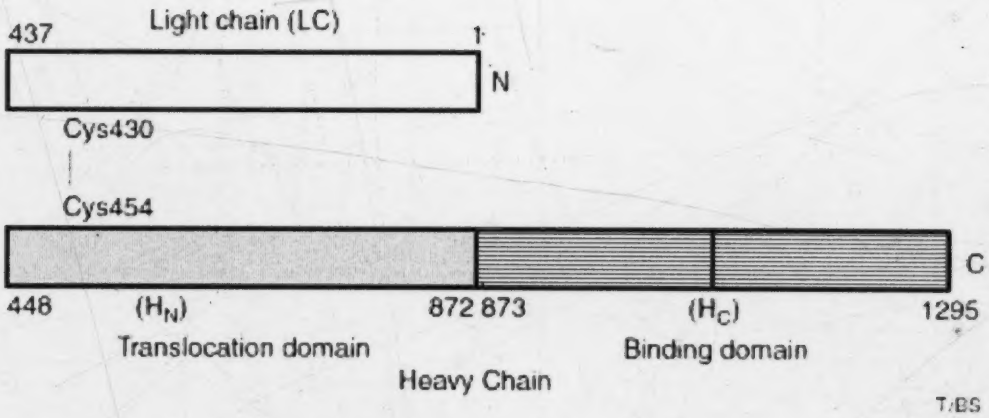

Figure 1.9. The di-chain structure of a Clostridial neurotoxin – botulinum neurotoxin A (BoNT/A). Clostridial neurotoxins (CNT) are ~150-kDa proteins, synthesized as single-chain polypeptides and post-translationally modified to form di-chain molecules. CNT share the same domain architecture and overall structure. The light and heavy chains of BoNT/A are linked by a single disulfide bond, Cys430–Cys454. The light chain (LC), shown in white, functions as zinc-dependent endopeptidase and contains the catalytic zinc atom and HExxH motif associated with zinc-dependent proteases. The heavy chain comprises two functional domains of roughly equal size. The N-terminal section (HN), shown in grey, is the translocation domain, which forms ion channels spanning endosomal membranes and is thought to be involved in translocation and activation of the LC. The C-terminal section (HC), (striped region), is the binding domain.
(Modified from: *Turton et al. Trends Biochem Sci. 2002 Nov;27(11):552-8.*)

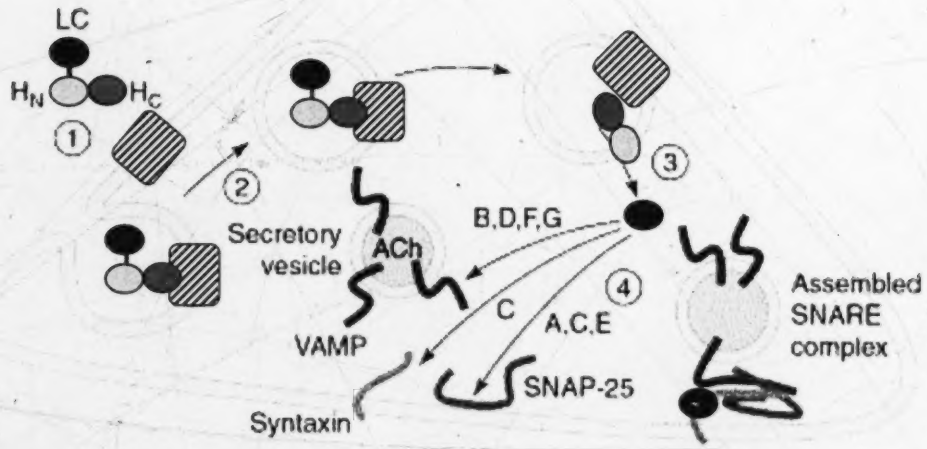

Figure 1.10. Mechanism of action of botulinum neurotoxins (BoNT). Each of the structural elements of botulinum neurotoxin, the light chain (LC) endopeptidase (black), the HN translocation domain (grey) and the HC cell-binding domain (horizontal striped) have a role in the mechanism of action of neurotoxin. The first stage in the intoxication process (interaction with gangliosides and an, as yet unidentified, protein receptor (striped square) (1) is followed by stage two, internalization of the toxin-receptor complex into an intracellular vesicle (2). The third stage (translocation) is characterized by release of the light chain endopeptidase from an acidic intracellular compartment into the cytosol (3). Once liberated from the vesicle, the light chain performs the final stage of intoxication; highly specific proteolytic cleavage of one of the proteins of the SNARE complex (4). BoNTs B, D, F and G cleave proteins of the VAMP family and BoNTs A, C and E cleave SNAP-25. BoNT/C also has the capacity to cleave syntaxin. Cleaved SNARE proteins are competent for facilitating docking of the secretory vesicle with the synaptic membrane, but fusion of the vesicle is compromised. Thus neurotransmitter release is inhibited. Abbreviations: HN, heavy chain N-terminal subdomain; HC, heavy chain C-terminal subdomain; SNARE, soluble NSF-attachment protein receptors; VAMP, vesicle-associated membrane protein; SNAP-25, synaptosomal-associated protein of 25 kDa.(Modified from: *Turton et al. Trends Biochem Sci. 2002 Nov;27(11):552-8*)

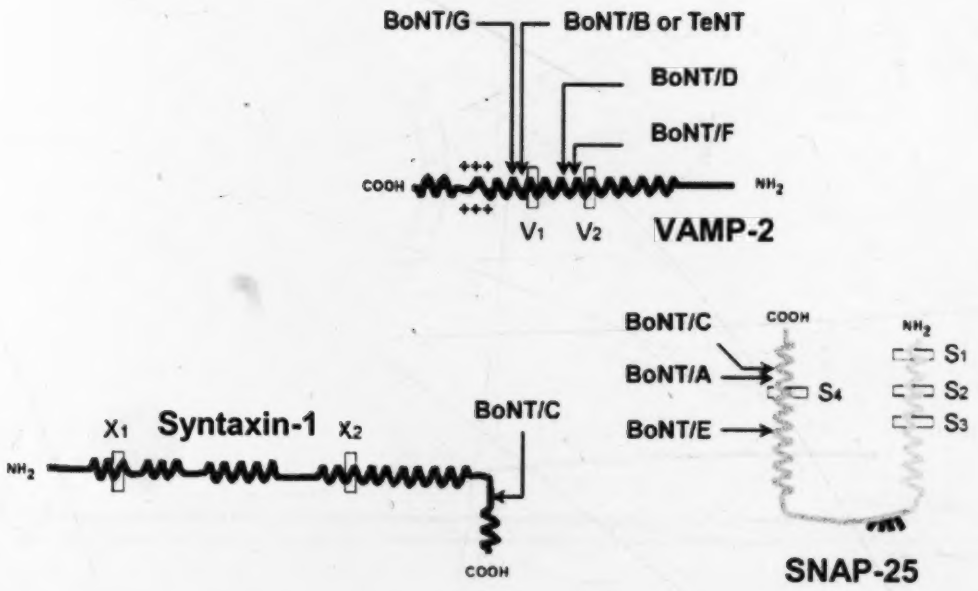

Figure 1.11. Binding and cleavage sites of CNTs. The three synaptic proteins cleaved by TeNT and BoNTs light chains are shown. The cleavage sites are indicated by arrows. The residues delimited by a box denote the sites that determine the binding specificity of the toxins light chains (also termed 'SNARE-binding motif'(not the same as the SNARE motif for complex assembly). An additional binding site for the TeNT light chain on VAMP, rich in positive charges is indicated (+++).
(Modified from : *Humeau Y. et al. 2000. Biochimie. 82(5) 427-446*)

II. STUDY OBJECTIVES AND HYPOTHESIS

The intracellular signaling molecules and mechanisms that regulate exocytosis of granule-derived mediators from eosinophils and neutrophils are poorly understood. The identification of proteins involved in these processes are critical to not only increasing our understanding of the pro-inflammatory role of these cells, but may provide novel therapeutic targets aimed at modulating secretory function. In accordance with the SNARE hypothesis,[245] the expression and localization of distinct SNARE isoforms may, in part, regulate the secretion of different granule compartments. Brumell et al (1995) first reported the expression of SNARE isoforms in human neutrophils. They showed that VAMP-2 in was localized to tertiary granules and small vesicles but was largely absent from secondary and primary granules by immunofluorescent and electron microscopy. The neuronal Q-SNAREs, syntaxin-1 and SNAP-25, were not detected. However, syntaxin-4 was found to be expressed almost exclusively at the plasma membrane.[322] A study by Lacy *et al* (1995) similarly found that neuronal SNARE isoforms, VAMP-1 and-2, SNAP-25 and syntaxin-1 were not detectable in subcellular fractions from guinea pig eosinophils.[323] However, follow up experiments in our laboratory demonstrated that VAMP-2 exhibited a punctate staining pattern in human eosinophils using confocal microscopy (P. Lacy. 1999).

Taken collectively, the above-mentioned two studies suggested that exocytosis of stored vesicle/granule mediators from eosinophils and neutrophils may be SNARE-dependent. In addition to neutrophils[322], syntaxin-4 was shown to be a candidate Q-

SNARE for exocytosis from human platelets.[312;324] The homologue of SNAP-25, SNAP-23[325], was also implicated in exocytosis from non-neuronal cell types such as mast cells and platelets.[312;326] I hypothesized that VAMP-2 was a critical R-SNARE for exocytosis from human eosinophils and neutrophils. Both syntaxin-4 and SNAP-23 were predicted to interact with VAMP-2 during granule/vesicle docking following exocytosis and to be essential for mediator release. In addition, it was anticipated that other isoforms of SNAREs and/or novel SNARE isoforms in these cells may impart specificity to exocytosis of specific granule compartments.

The research objectives of this project were two-fold: (i) To determine the expression and localization of candidate SNARE isoforms involved in exocytosis and (ii) to determine their functional role of SNARE isoforms in the release of stored granule mediators by antibody and/or CNT-mediated inhibition in permeabilized cells. The major findings of the study were as follows:

(1) VAMP-2 was identified as a candidate v-SNARE in eosinophil PMD and was localized to small vesicles, but not crystalloid granules. VAMP-2 was similarly detected in vesicle and membrane-enriched fractions but not on large azurophilic granules in human neutrophils. Neutralization of VAMP-2 function by BoNT-B or neutralizing antibodies caused a partial impairment of secretion in eosinophils, consistent with a role in PMD. In contrast, VAMP-2 inhibition did not significantly block mediator release from azurophilic, secondary or tertiary granules, suggesting it may be limited to small vesicle release in these cells.

(2) The t-SNAREs, SNAP-23 and syntaxin-4, were expressed on plasma membranes of eosinophils and PMNs. SNAP-23 and syntaxin-4 were found to interact in a *cis*-

SNARE complex in granulocytes. Trans-SNARE complexes representing VAMP-2 pairing with SNAP-23/syntaxin-4 were not detected, which may have been due to either epitope availability of the immunoprecipitating antibodies and/or rapid disassembly and recycling of trans-SNARE complexes. Neutralization of syntaxin-4 by specific antibodies modestly impaired the release of multiple granule mediators from human eosinophils and neutrophils.

(3) The neurotoxin-resistant R-SNARE isoforms, tetanus-insensitive VAMP (TI-VAMP) and/or VAMP-8 (endobrevin), were expressed on crystalloid granules and azurophilic granules, in addition to membrane-enriched fractions. Antibody-mediated neutralization of TI-VAMP potently impaired the release of stored mediators from both eosinophils and neutrophils, whereas VAMP-8 antibodies had not effect. TI-VAMP is largely expressed on lysosomes and/or late endosomes in other non-neuronal cell types. The observation that TI-VAMP is a crticial isoform for exocytosis of large lysosome-like crystalloid and azurophilic granules suggests that trafficking molecules previously identified in lysosome biogenesis and protein sorting may also be involved in granulopoeisis and/or exocytosis in granulocyte lineages.

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CHAPTER 2

METHODS AND MATERIALS

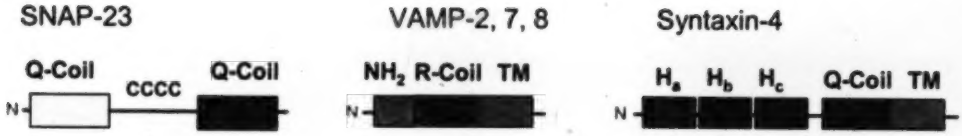
A. Materials

(i) Antibodies

SNAREs SNARE antibodies used and their recognition sites are summarized in Figure 2.1, *page 91*. Sodium azide (NaN_3)-free mouse mAb (IgG_1) to the cytoplasmic domains of human syntaxin-4 (aa 1-280) and rat syntaxin-6 (aa 1-136) were obtained from BD Pharmingen (San Diego, CA, USA). Mouse VAMP-2 mAb (Cl 69.1) (rat VAMP-2: aa 2-17) and rabbit anti-SNAP-23 antiserum (C-terminus peptide: DRIDIANARAKKLIDS) were obtained from Synaptic Systems, GmbH (Göttingen, Germany). Affinity-purified rabbit anti-SNAP-23 was also generously contributed by Dr. D. Castle (University of Virginia Health Sciences Center, Charlottesville, VA). Mouse mAb to human TI-VAMP clone 158.2 (aa 119-188) [1] and affinity purified anti-human VAMP-8 (TG15) rabbit IgG (N-terminus: MEEASGSAGNDRVRN) [2] were kind gifts from Dr. T. Galli (INSERM, Paris, France).

Other antibodies Mouse anti-CD63 mAb (IgG_1) was purchased from BD Pharmingen. Mouse IgG_1 and purified rabbit IgG were obtained from R&D Systems (Minneapolis, MN, USA) and Cedarlane Laboratories (Hornby, ON, Canada), respectively. Mouse anti-human MBP (BMK-13) is an in-house mAb and has previously been described.[3] CD9 antibodies were a gift from Dr. Andrew Shaw (Cross Cancer Institute, University of Alberta). Mouse monoclonal anti-calnexin

(IgG_1), a marker for endoplasmic reticulum (ER), was obtained from StressGen Biotechnologies (Victoria, BC, Canada). Rabbit polyclonal anti-giantin, a marker for *cis*-Golgi, was a kind gift from Dr. T. Hobman (Department of Cell Biology, University of Alberta). Rhodamine-Red-X-conjugated affinity purified donkey anti-rabbit IgG and Rhodamine-Red-X-conjugated affinity purified goat anti-mouse IgG were from Jackson Immunoresearch Laboratories Inc.(Westgrove, PA, USA) BODIPY FL labeled goat anti-mouse antibodies were purchased from Molecular Probes (Eugene, OR, USA). Phycoerythrin conjugated (PE) anti-rabbit and anti-mouse antibodies were purchased from Cedarlane laboratories (cat # CLCC43004, CLCC30004).



Antibody	Region	Recognizes
Anti-VAMP-2	NH_2 domain	monomeric protein
Anti-VAMP-7	NH_2 & coil	monomeric protein
Anti-VAMP-8	NH_2 domain	monomeric protein
Anti-syntaxin-4	H_{abc}, coil	monomeric & complexed protein
Anti-syntaxin-6	H_{abc}, coil	monomeric & complexed protein
Anti-SNAP-23	C term. coil	monomeric & complexed protein

Figure 2.1. SNARE domains and antibody recognition sites. The amino-terminal domain of syntaxins, (including syntaxin-4) form a three-helix bundle (red) that binds to its carboxy-terminal coil domain (purple), forming a closed conformation (see also Figure 1.6, *page 42*). One "Q"coil domain of syntaxin combines with 2 "Q" coils SNAP-23 and 1 "R" coil from either VAMP-2, 7 or-8 to form the four-helix SNARE complex. The NH_2 terminal 3-helix coil of syntaxin (Habc domain) does not participate in SNARE-SNARE interactions, but extends out of the SNARE complex (see Figure 1.5, *page 41*) (TM, transmembrane domain) SNAP-23, like SNAP-25 is anchored via four palmitoylated cysteine residues (CCCC). Antibodies used and their recognition regions are shown.
(Modfied from: *Chen YA & Scheller RH. 2001. Nature Rev. Mol. Cell Biol.* **2** 98-106)

(ii) Other reagents

Streptolysin-O (SLO) (100 µg/ml stock) was purchased from Dr. S. Bhakdi (Johannes Gutenberg University, Mainz, Germany). *Botulinum* B light chain (BoNT-B LC) and tetanus holotoxin (TeNT) were purchased from Calbiochem (LaJolla, CA, USA). Nycodenz was purchased from Invitrogen (Carlsbad, CA, USA).

B. Preparation of eosinophils and neutrophils

Isolation of eosinophils and neutrophils from atopic and healthy individuals, respectively, has been described previously.[4] Briefly, erythrocytes from peripheral blood (100ml: eosinophil isolations or 50 ml: neutrophil isolations) were sedimented in 6% dextran (10 ml 6% dextran to 40ml whole blood) for 30 min. Leukocyte-rich supernatants were collected and layered onto Ficoll-paque (Amerham Biosciences, PQ, Canada) (50ml blood / 15ml Ficoll gradient) and centrifuged for 25 min at 1200 rpm (~350*g*) at room temperature. Monocytes at the Ficoll interface were removed and contaminating erythrocytes in granulocyte pellets lysed in 0.5 ml water and washed in RPMI-1640 (with HEPES modification) containing 5 mM EGTA (Buffer A). Cells were resuspended in Buffer A + 10% FBS for further applications. Neutrophil purity was >98%. For eosinophil isolations, eosinophil/neutrophil granulocyte pellets were resuspended at 1 x 10^6 cells/ml and incubated for 30 min at room temperature with CD16 immunomagnetic beads (12 µl beads/ 5 x 10^7 cells), CD3 and CD14 beads (10 µl of each) Miltenyi Biotec Inc.(Auburn, CA, USA). Highly purified $CD16^-$ eosinophils (>99%) were isolated by immunomagnetic selection using AutoMACS, Miltenyi Biotec Inc.(Auburn, CA, USA).

C. RT-PCR, subcloning and sequencing of PCR products

RNA was extracted from granulocytes (2×10^6 cells minimum/extraction) with Trizol reagent (Invitrogen) producing 0.2–1 μg RNA. Cells were solubilized in Trizol (1 ml / 5-10 x 10^6 cells) and incubated for 5 min. A volume of 0.2 ml chloroform per 1 ml Trizol was added and tubes shaken vigourously for 15 sec. Tubes were centrifuged at 12,000*g* for 15 min at 4C. The aqueous supernatant was collected and transferred to a clean tube. 0.5 ml isopropanol was added/ 1 ml Trizol used initially and tubes incubated 10 min at room temperature. Tubes were centrifuged at 12,000*g* for 10 min at 4 °C. The RNA pellet was washed once in 75% ethanol and pelleted by centrifugation at 7,500*g* for 5min. Pellets were dried under vacuum for 30-60 min, resuspended in RNase-free water (10 μl/sample) and stored at –80 °C until use.

Reverse transcription (RT-PCR) was carried out as follows: 1 μg total RNA (or less) was added to 1 μl oligo-$(dT)_{12\text{-}18}$ (500 μg/ml) and topped to 11 μl with RNase free water. The mixture was heated for 10 min at 70 °C, following which 8 μl of RT master mix was added per reaction (4μl 5X First stand buffer (Gibco/BRL), 2μl 0.1 M DTT, 1 μl mixed dNTPs (10 mM), 1 μl RNase-free water). 1 μl of M-MLV (200 units) was added and reactions incubated for 37 C for 1 hr. Reactions were then terminated at 70 °C for 10 min. cDNA generated was stored at –20 °C or used immediately for PCR reactions.

PCR reactions using intron-spanning primers for the housekeeping gene β2-microglobulin and SNAREs was performed. Primer sequences were directed human sequences of VAMP-2 (NIH Genbank Access. No. NM_014232.1), TI-VAMP

(NM_005638), VAMP-8 (NM_003761), syntaxin-4 (AF026007), syntaxin-6 (NM_005819) and SNAP-23 (U55936) Primer sequences were as follows:

VAMP-2: forward nt- 5' ATGTCTGCTACCGCTGCCACG-3'

reverse nt – 5' AGAGCTGAAGTAAACTATGATGATG-3'

TI-VAMP: forward-nt-70-89 5' AGACTGAAGCCATGGCGATT-3'

reverse-nt-721-742-5'-CTATTTCTTCACACAGCTTGGC-3'.

VAMP-8: forward-nt-14-33 5'-GGCGAATTCACTTACTGACC-3'

reverse-nt-581-600 5'-GTCTCTCCAGCCCACTCTAA-3'.

Syntaxin-4: forward nt-5'-ATGCGCGACAGGACCCACGAGCTG-3'

reverse nt-5'-TTATCCAACCACTGTGACGCCAATGAT-3'

Syntaxin-6 forward-nt-16-35 5'- CCCTTCTTTGTGGTGAAAGG -3'

reverse-nt-545-564 5'- CTGGGACATGTTCTTCAGCA -3'.

SNAP-23: forward nt-5'-CTGGGTTTAGCCATTGAGTCTCAGG-3'

reverse nt- 5'-GGTGTC AGCCTTGTCTGTGATTCG-3'

Primers for VAMP-2[182], SNAP-23[19] and syntaxin-4[182] were from previously published reports. All other primers were designed using the Primer3 primer design program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Specificity and template alignment of primers was confirmed using BLAST (NCBI) and Clustal X software.

PCR reactions were carried out in 20 µl volume according to the following protocol. 2 µl of cDNA was added to 17.5 µl of PCR master mix. 0.5 µl of Taq polymerase was added immediately before amplification. Reactions were carried out in a PTC 100 Thermal Controller (M-J Research) according to the following program:

Step 1: 5 min at 94 °C, Step 2: 45 sec at 94 °C, Step 3: 45 sec at 60 °C, Step 4: 2 min at 72 °C, Step 5: repeat Steps 1-4 for 30 cycles, Step 6: 7 min at 72 °C, Step 7: 4 °C. Amplified products were subcloned and sequenced as previously described.[5] Briefly, PCR products were subcloned into the pCR™ 2.1 plasmid vector using the TIA cloning kit (Invitrogen Corporation, Carlsbad, CA) and double-stranded plasmid DNA isolated with the Wizard Plus Mini-prep kit (Promega Corporation, Madison, WI) as previously described.[5] Plasmid DNA, containing the inserted PCR product, was sequenced using M13 forward and reverse primers and carried out on an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA)

D. Confocal laser scanning microscopy (CLSM)

Eosinophils were resuspended at 1 x 10^6 cells/ml in color-free RPMI 1640 containing 20% FBS and cytospins generated by centrifugation at 400 rpm (50, 000 cells/slide). Slides were air dried for 1 h, wrapped in aluminum foil and stored at -20 °C until use. Slides were warmed to room temperature, re-hydrated for 1 min in PBS, then fixed in 2% paraformaldehyde (in PBS) for 5 min. Slides were washed in TBS, and blocked for 0.5 hr in 5% goat serum in TBS.

(i) Single labeling for t-SNAREs Slides were incubated for 1 hr with TBS containing 2.5 μg/ml affinity purified anti-SNAP-23, 12.5 μg/ml anti-syntaxin-4, 5 μg/ml anti-calnexin, or anti-giantin (1:3000). Purified rabbit IgG (2.5 μg/ml) was used as the negative control for anti-SNAP-23, normal rabbit serum (1:3000) was used as the control for anti-giantin, and mouse IgG_1 (12.5 μg/ml and 5 μg/ml) was used as the control for anti-syntaxin-4 and anti-calnexin, respectively. SNAP-23 and giantin (as *cis*-Golgi marker) immunoreactivity were detected using 10 μg/ml Rhodamine-Red-X-

conjugated affinity purified donkey anti-rabbit IgG. Syntaxin-4 and calnexin immunoreactivity were detected using 14 μg/ml Rhodamine-Red-X-conjugated affinity purified goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc.). Images were captured using the Molecular Dynamics Confocal Imaging System 2001 (Molecular Dynamics, Sunnyvale, CA).

(ii) *Double labeling of VAMP-2 and RANTES* Slides were incubated for 1 hr with 5 μg/ml anti-VAMP mAb or isotype control (mIgG1), washed 3X in TBS and labeled with 20 μg/ml BODIPY FL secondary antibodies. Slides were washed and blocked for 1 h in 10 μg/ml goat anti-mouse Fab fragment (Jackson Immunoresearch Inc.), following which they were incubated for 1 h with anti-human RANTES mAb (R&D Systems) at room temperature. Slides were washed in TBS and immunoreactivity to RANTES detected by incubation with 15 μg/ml goat anti-mouse IgG conjugated to TRITC or Rhodamine Red (Jackson Immunoresearch Inc.) Confocal analysis was carried out on labeled cells using the Molecular Dynamics Confocal Imaging System 2001 (Molecular Dynamics, Sunnyvale, CA) and Image Space 3.2 software for detection and imaging of immunofluorescence. The pixel intensity of the cytoplasmic regions of the cells was quantified by positioning an arbitrary fixed circle (124 pixels) over randomly selected areas within the cytoplasm. Average values and standard errors of the mean were achieved for the time course (between 2-11 cells/time point) by measuring between 15-89 cytoplasmic regions of 124 pixels at each time point. Cell profiles were collected by plotting pixel intensities obtained from the radius (drawn from the cell center to perimeter) of representative cells. Values were averaged and their standard errors of the mean computed. Results were analyzed for

significance using Student's t test (one-tailed) for flow cytometry, and Kruskal-Wallis one-way analysis of variance (together with Dunn's multiple comparison test) for data obtained using confocal analysis. Results were considered significant when $p < 0.05$.

E. Subcellular fractionation using a linear nycodenz gradient

A detailed description of reagents and the protocol for subcellular fractionation is described in Appendix A. Cells were washed (2X) in ice cold Buffer A and resuspended at 15×10^6 cells/ml (eosinophils) or 20×10^6 cells/ml (neutrophils) in ice cold Buffer B. Cells were homogenized using a precision ball-bearing cell cracker (EMBL, Heidelberg, Germany) using 8.010 mm ball bearing (~15-20 passages) and 3 cc syringes. Homogenization was confirmed by examination for intact cells by light micoscopy (40X objective). Nuclei from homgenates were removed by centrifugation for 10 min at 2000 rpm (450*g*) in a Sorvall centrifuge. Post-nuclear supernatant was layered on top of a linear Nycodenz (0-45%) gradient and centrifuged for 1 h at 100, 000*g* (30, 000 rpm) using a SW 40TI rotor in a Beckman ultracentrifuge (brake and acceleration set at minimum). Fractions were collected using a fraction collector (18 drops/fractions for neutrophils; 24 drops/fraction). Fractions were aliquoted (3X) and one set of 1:10 diluted fractions made (50 µl of fraction : 450 µl Buffer A). Fractions were stored at –80 ^{0}C until use for marker assays or Western blot analysis.

F. Marker assays for subcellular fractions

Fractions were examined for activities of marker proteins: Peroxidase, EPO and MPO, markers of eosinophil CG [6] and neutrophil AG [7], respectively; β-hexosaminidase (B-HEX) (marker of eosinophil small granules[6] and azurophilic

granules [7;8]), lactate dehydrogenase (LDH) (a cytoplasmic marker [9]). Membrane-enriched fractions from eosinophils were detected by dot-blot analysis for CD9. Fractions enriched in neutrophil secretory vesicles/plasma membranes were detected by alkaline phosphatase activity in the presence or absence of 0.1 % Triton-X-100, based on previous studies.[10;11]

(i) EPO and MPO assay Eosinophil peroxidase (EPO) and neutrophil myeloperoxidase (MPO) were detected using 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma, Oakville, ON, Canada).[12] A volume of 50 µl of 1:10 diluted fractions were added to 150 µl of TMB substrate and incubated 10-15 min at room temperature. Reactions were terminated by the addition of 50 µl of 1M H_2SO_4 and read at 450 nm in colorimetric plate reader. The percentage release of maximum calculated according to the following formula: $[(OD_{test} - OD_{blank}) / (OD_{max\ test\ value} - OD_{blank})] \times 100$.

(ii) β-hexosaminidase (β-HEX) assay A total of 50µl of 1:10 fractions were added to 50 µl of β-HEX substrate solution (37. 95 mg of 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma M-2133) dissolved in 1 ml dimethylsulfoxide (DMSO) added to 99 ml 0.2 M citrate buffer, pH 4.5) and incubated for 2 h at 37 ^{0}C. Reactions were terminated by addition of 100 µl of 0.2 M Tris buffer. Plates were read at 450 nm (excitation 356 nm) in a fluorescent plate reader. The percentage release of maximum calculated according to the following formula: $[(OD_{test} - OD_{blank}) / (OD_{max\ test\ value} - OD_{blank})] \times 100$.

(iii) LDH assay I added 10 µl of neat fractions to 80 µl of reaction mixture (1 mg/ml β-NADH (Sigma) in 0.75 M pyruvate, pH 7.5) and incubated for 30 min at 37 C.

Following incubation, 80 µl of dinitrophenylhydrazine solution (100 µl of 20 mg/ml 2, 4- dinitrophenylhydrazine in DMSO added to 10 ml of 1M HCl) was added per well and incubated for 20 min at room temperature. The reaction was stopped by addition of 3.5 M NaOH and plates read at 450 nm. The percentage release of maximum calculated according to the following formula: ABS (absolute value)[(OD_{test}– OD_{blank}) / ($OD_{max\ test\ value}$ – OD_{blank})] x 100. (For this assay, lower absorbance values correlate with higher LDH activity).

(iv) Alkaline phosphatase assay (neutrophil fractions) I added 30 µl of neat fractions to 120 µl of substrate solution (200 mg of p-Nitrophenyl phosphate disodium salt (Sigma 104 Phosphate substrate Mw=263.1 in 100 ml 0.2 M Tris, pH 10.5 containing 5 mM $MgCl_2$) with or without 0.1% TX-100 to determine peak activities of secretory vesicles and plasma membranes, respectively. Reactions were incubated for 30 min at 37 °C and terminated with 100 µl of 2 M NaOH. Plates were read at 410 nm and the % release of maximum calculated according to the following formula: [(OD_{test}– OD_{blank}) / ($OD_{max\ test\ value}$ – OD_{blank})] x 100.

(v) CD9 dot blot analysis (eosinophil fractions) All incubations were performed at room temperature. Neat fractions were spotted onto of onto nitrocellulose strips (~1 cm spacing/fraction, 2 µl/spot) and allowed to dry for 15-30 min. Strips were placed in incubation trays and blocked for 20 min in 5% milk in TBS-0.1% Tween 20 (TBS-T), pH 7.6. Blocking solution was removed and strips incubated for 30 min in CD9 mAb (1:1000 in 5% milk in TBS-T). Strips were washed (2X) for 15 min in TBS-T. Rabbit anti-mouse antibodies (DAKO Diagnostics Canada, Mississauga, ON, Canada) were added for 30 min (1:40 in 5% milk TBS-T). Strips were washed (2X) for 15 min in

TBS-T. Mouse APAAP (DAKO Diagnostics Canada) was added at 1:30 (in 5% milk in TBS-T) for 30 min. APAAP was removed by 2X washes for 15 min in TBS-T. Alkaline phosphatase reagent (1 mg/ml Fast Red Sigma F2768) was added for a maximum of 15 min and then strips rinsed with ddH2O for 10 min. Strips were dried, scanned and immunoreactive "dots" quantitated. Pecentage of maximal activity was expressed according to the formula: $(Density_{test}) / (Density_{max\ test\ value}) \times 100$.

G. Generation of high-speed light membrane pellets

Cells were homogenized using a precision ball-bearing cell cracker and post-nuclear supernatant generated as described in the subcellular fractionation section. Granules were pelleted from post-nuclear supernatants by centrifuation at 10, 000*g* for 10min. Supernatants containing cytosol and light membranes (secretory vesicles, plasma membranes, ER membranes and Golgi membranes) were stored at –80 ^{0}C until use. These supernatants were (occasionally) pooled from separate donors and centrifuged at 100, 000*g* (32, 000 rpm) for 1 h in a SW 50.1 rotor in a Beckman ultracentrifuge. Membrane pellets were either resuspended in 3X SDS loading buffer for Western blot analysis or 0.5 ml RIPA buffer (PBS + 1% Nonidet P-40 + 0.1% SDS) containing a protease inhibitor cocktail (5 µg/ml each of leupeptin, aprotinin and TAME (Sigma)) for immunoprecipitation experiments (see below).

H. Digestion of VAMP-2 in light membrane pellets using tetanus toxin (TeNT)

Lyophilized tetanus holotoxin (TeNT) was resuspended in 0.1 M KH_2PO_4, pH 7.4, supplemented with 0.1% BSA. Holotoxin was reduced by incubation in 0.01 M DTT for 30 min at 37°C. A sample of reduced toxin was denatured by boiling the toxin for

10 min. Reduced toxin and denatured toxin (225 ng) were each added to 5 x 10^6 cell equivalents of eosinophil light membrane pellets in 2 mM $ZnCl_2$ and 10 mM DTT. Samples were incubated for 1 h at 37°C, and transferred to ice to stop the reaction. SDS-PAGE and Western blot analysis was carried out using mouse monoclonal antibody to VAMP-2 (1:10,000)

I. Western blot analysis and immunoprecipitation

(i) Western blot analysis

Subcellular fractions representing peak marker activities for granules, membranes/vesicles, and cytosol were subjected to SDS-PAGE (16% acrylamide content) (25µl fraction/lane: 0-10 µg maximum protein/lane) and Western blot analysis. Samples were resuspended in an equal volume of 3X SDS loading buffer (New England Biolabs, Beverly, MA, USA) and boiled for 3 min.[13] Lysates of human platelets, rat brain and CaCo-2 cells were used as positive controls for SNARE protein expression (rat brain: VAMP-2, TI-VAMP, syntaxin-6; the human colonic cell line, CaCo-2: VAMP-8; platelets: SNAP-23 and syntaxin-4). Whole rat brain was homogenized in ~ 2-4 ml of RIPA buffer using a dounce homogenizer. Homogenate was incubated on ice for 0.5 hr and nuclei/tissue debris removed by centrifugation at 7,000*g* for 10 min. Homogenates were aliquoted and stored at –80 ^{0}C until use. containing 5 µg/ml protease inhibitor cocktail (5 µg/ml leupeptin, aprotinin and TAME (Sigma)) For preparation of platelet lysates, peripheral blood (50 ml) was obtained from mildly atopic donors, and subjected to centrifugation at 300*g* for 10 min. Plasma-rich supernatant devoid of cells was harvested and centrifuged at 1800*g* for 5-8 mins, and the platelet-rich pellet resuspended in 1 ml 0.25 M sucrose, 10 mM

HEPES, pH 7.4, and 1 mM EGTA containing 0.1% Triton X-100. CaCo-2 cells were provided by Dr. Glen Armstrong (University of Calgary). Cells were homogenized at 20 x 10^6 cells/30µl RIPA buffer and left on ice for 0.5 h. Nuclei were removed by centrifugation at 7,000*g* for 10 min and lysates stored at –80 °C until use. Protein content of lysates was determined using the *DC* assay (Bio-Rad Laboratories, Mississauga, ON, Canada).

Protein from electrophoresed gels were transferred to Immobilon PVDF membranes (Millipore, Bedford, MA, USA) using a semi-dry transfer (BioRad Laboratories) for 45 min at 15 V. Immobilon blots were incubated in 5% milk in TBS-T either overnight at 4C or for 1 h. Primary antibodies were diluted in 5% milk in TBS-T with the exception of TI-VAMP antibodies (these must be diluted in TBS-T alone without milk) and incubated for 1 h at room temperature. Dilutions for antibodies were as follows: VAMP-2 mAb: 1:10, 000 (50 ng/ml); TI-VAMP mAb: 1:1000; VAMP-8 antiserum: 1:1000; syntaxin-4 mAb: 1:5000 (50 ng/ml); syntaxin-6 mAb: 1:1000 (250 ng/ml); SNAP-23 antiserum: 1:1000. Blots were washed 2X in TBS-T for 15 min. Secondary antibodies conjugated to horse-radish peroxidase, goat anti-mouse HRP and donkey anti-rabbit HRP (Amersham BioSciences, Baie d'urfe, PQ, Canada) were added (1:5000) for 1 h at room temperature. Blots were washed 4 X 5 min in TBS-T. ECL substrate was added (Pierce SuperSignal, MJS Biolynx Inc., Brockville, ON, Canada) for 1 min to blots and exposed to Hyperfilm (Amersham Biosciences) for 5-30 min exposure time.

(ii) ***Immunoprecipitation of syntaxin-4 from high speed light-membrane pellets***

For co-precipitation experiments, high speed light-membrane pellets were resuspended in 0.5 RIPA buffer containing 5 μg/ml protease inhibitor cocktail (5 μg/ml each of leupeptin, aprotinin and TAME (Sigma)). Resuspended pellets were precleared with 2.0 μg mouse IgG1 and 40μl of a 1:1 slurry of RIPA buffer: Protein G Sepharose beads (Amersham Biosciences) for 1 h at 4 ^{0}C with continuous rotation. Beads were pelleted by centrifugation at 1,000*g* for 10 min. Supernatants were transferred to clean tubes and incubated with either 2.0 μg anti-syntaxin-4 mAb or 5 μl of SNAP-23 for 1 h (minimum) or overnight with continuous rocking. 40 μl of 1:1 bead slurry (RIPA buffer: Protein G Sepharose) was added for 1 h at 4°C and beads pelleted by centrifugation at 1,000*g* for 10 min. Precleared beads and immunoprecipitate beads were washed 3X in RIPA buffer, resuspended in an equal volume of 3X SDS loading buffer, boiled for 3min and subjected to acrylamide gel electrophoresis (16% acrylamide content).

J. FACS analysis of granule fractions

Granules from subcellular fractions exhibiting peak EPO and MPO activities were pelleted by centrifugation at 12,000*g* for 15 min. Granule pellets were fixed in 5% formalin/PBS, pH 7.2 for 10 min, and washed twice in cold PBS. Fixed granules were blocked in 5% milk/PBS for 1 hr or overnight. Granules were washed in PBS and incubated with primary antibodies: 1:200 (5 μg/ml) VAMP-2 mAb, 1:100 TI-VAMP, 1:100 VAMP-8 antiserum, 1:50 syntaxin-6 mAb (5 μg/ml) in 5% milk/PBS for 1 h. Permeabilized eosinophil granules and non-permeabilized neutrophil granules were examined for the expression of CG and AG granule-membrane markers, MBP

and CD63, respectively. For MBP labeling of eosinophil granules (positive control), blocking and antibody incubations were performed under permeabilizing conditions (+ 0.1% saponin) (1:100 anti-MBP mAb). Neutrophil granules were labeled with 1:200 anti-CD63 mAb (positive controls) under non-permeabilizing conditions. Following primary antibody incubation, granules were washed three times in PBS and incubated for 1 h in 1:200 PE-conjugated secondary antibodies (Cedarlane). Granules were washed three times in PBS and examined by FACS analysis for mean fluorescence intensity (MF1) the entire cell or granule population (ungated). Percentage of positive events determined in respect to isotype control antibodies (% gated in M1). Results from three separate experiments were averaged, the SEM calculated, and significance from isotype controls ($p < 0.05$) determined using a paired T-test.

K. Ca^{2+} and GTPγS-induced mediator secretion in SLO-permeabilized cells

Rationale, background and experimental protocol

The exogenous application of Ca^{2+} and GTPγS was previously demonstrated to activate secretion in permeabilized eosinophils and neutrophils.[14] Since mature end-differentiated cells such as eosinophils and neutrophils are not easily transfected with recombinant proteins, we selected to modify the permeabilization technique described by Gomperts and colleagues [15] to introduce SNARE neutralizing antibodies into permeabilized cells. The targeting of SNARE isoforms by SLO permeabilization has been successfully used for other non-neuronal cell types. [16] In addition, permeabilization or microinjection is required to examine the effects of clostridial

neurotoxins, such as TeNT and BoNT, in non-neuronal cell types, as exogenous toxins selectively bind and enter neurons only.[17;18]

It was previously demonstrated that the intensity of the secretory response was dose-dependent on calcium.[14] In order to be able to detect inhibitory responses using SNARE antibodies we wished to establish the maximal secretory response using an optimal dose of SLO and calcium which would not compromise cell integrity. We utilized a series of EGTA-buffered calcium solutions, described previously [15], to determine the maximal secretion of EPO from human eosinophils. The EPO assay (described above) was selected for these optimization experiments since it is fast, quantitative and cost-effective. A detailed description of materials and methods for SLO permeabilization and activation of the cells with Ca^{2+} and GTPγS is described in Appendix A.

Briefly, cells were washed 3 times in BSS (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 20 mM PIPES, pH=6.8) and resuspended at 1 x 10^6 cells/ml. Cells were added in triplicate to v-well plates (50,000 cell/well) and permeabilized in 0.1-1.0 μg/ml streptolysin-O (SLO) for increasing periods of time (0, 1, 2, 5, 10, 15 min). Exocytosis was induced by the addition of Ca^{2+} and GTPγS using Ca^{2+} EGTA-buffered BSS. Granulocytes were incubated in either pCa 5, 5.5, 6.5, 7 supplemented with 10 μM GTPγS (activated) or pCa 7 in BSS alone (negative control) (note: pCa X= 10^{-x} M Ca^{2+}). Reactions were terminated at 10 min by the addition of 100 μl cold BSS, plates centrifuged at 350g for 5 min at 4°C, and supernatants examined for EPO activity (see protocol above). Cell lysates (using 0.5% Triton-X-100) were used to determine total peroxidase activity. Values were expressed as either percent of total peroxidase

activity (detected in lysates) or % of maximal release (detected at pCa 5) according to the following formulae:

% total peroxidase: $[(OD_{test} - OD_{\text{neg control (pCa 7)}}) / (OD_{\text{lysed}} - OD_{\text{neg control (pCa 7)}})] \times 100$

% maximal release: $[(OD_{test} - OD_{\text{neg control (pCa 7)}}) / (OD_{\text{pCa5}} - OD_{\text{neg control (pCa 7)}})] \times 100$

Results obtained from optimization experiments

The secretion of EPO from permeabilized human eosinophils was elevated in response to increasing Ca^{2+} doses (Figure 2.2A, *page 111*). A maximum secretion of 58% ± 14% of total peroxidase (EPO) activity was obtained at pCa 5 (n=5). A minimum dose of 1.0 μg/ml SLO was required to induce this response from eosinophils and was used for all subsequent experiments using SNARE antibodies. Neutrophils were similarly found to exhibit Ca^{2+}-dependent release of MPO (68% ± 8% of total at pCa 5) in optimization experiments (data not shown). In contrast to eosinophils, neutrophils were readily permeabilized at lower doses of SLO (0.1 μg/ml minimum effective dose). The doses of SLO used for these experiments are comparable to previously reported effective doses, which are known to vary according to cell type, [19] [20] and were used for all subsequent secretion experiments. [21;22] "Negative controls" (permeabilized at pCa 7) exhibited a small spontaneous release values of EPO and MPO, 9% ± 3% and 14% ± 5%, from eosinophils and neutrophils, respectively.

Eosinophils and neutrophils exhibited a decrease in the secretory response of EPO and MPO, respectively, at increasing incubation times with SLO prior to activation. This "rundown" of the maximal secretion response was previously described in studies by Gomperts and colleagues, and is anticipated to reflect the

increasing loss of cytosolic signaling proteins following permeabilization.[19;23] Delayed addition of Ca^{2+} and GTPγS to eosinophils markedly reduced the secretion of EPO as early as 5 min, while neutrophil secretion exhibited a more modest impairment which was not evident until ~10 min (Figure 2.2B, *page 111*).

A previous study from our laboratory demonstrated that eosinophils rapidly (within 10 min) and selectively released RANTES following treatment with IFN-γ. RANTES was localized to both small secretory vesicles and the matrix of eosinophil crystalloid granules.[6] Stimulation with IFN-γ caused the selective release of RANTES as indicated by the loss of RANTES but not MBP immunoreactivity by confocal laser scanning microscopy (CLSM). RANTES secreted from eosinophils, at a density of 4 x 10^6 cells/ml was determined by ELISA to be ~80 pg/ml (maximum) at ~2 h. IFN-γ-induced secretion of RANTES from eosinophils was confirmed in a separate study by Bandeira-Melo *et al* using a microscopy-based fluorescent assay.[24] We hoped to examine RANTES secretion from SLO-permeabilized eosinophils in the presence or absence of SNARE antibodies. In optimization experiments using densities of eosinophils 1-2 x 10^6 cells/ml only small quantities of RANTES were released (~30-50 pg/ml) following stimulation with pCa 5 and 10 μM GTPγS. Similar results were obtained using 500 IU IFN-γ as a stimulus in SLO-permeabilized eosinophils (data not shown). Since the lowest detection limit of the RANTES ELISA kit is near these values (30 pg/ml) (R&D systems) and quantitative measurements would be required for experiments using SNARE antibodies, RANTES was not examined from permeabilized cells. It is possible that increasing the density of eosinophils may permit the measurement of the relatively small amount of RANTES

secreted from eosinophils. However, due to the limited eosinophil harvest from 100 ml peripheral blood and the necessity for adequate controls and replicates, increasing cell density was not possible for these experiments.

Protocol for SNARE antibody incubations

To examine the role of SNARE isoforms, cells were permeabilized in SLO in the presence of SNARE-specific Abs (0.1 –20 μg/ml) or matched isotype control mouse IgG_1 or rabbit IgG from R&D Systems (Minneapolis, MN, USA). To ensure that permeabilized granulocytes exhibited near maximal secretory responses, cells were treated with SLO in the presence of control or SNARE antibodies for 2 min at 37°C prior to activation with Ca^{2+} (pCa5) and GTPγS for 10 min. As shown in Figure 2.3, *page 112* (see methods below) (also Figure 5.5, Chapter 5), this short permeabilization period was sufficient to permit the entry of exogenous antibodies into SLO-permeabilized cells.

L. Analysis of granule-derived mediator release from SLO-permeabilized cells

Neutrophil supernatants were examined for myeloperoxidase (MPO) (marker for AG), lactoferrin (LTF) (marker for SG) and matrix metalloproteinase-9 (MMP-9) (marker for TG).[25] Eosinophil supernatants were examined for secretion of eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN).[26;27] Release of EPO and MPO was determined on fresh undiluted supernatants using 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma, Oakville, ON, Canada).[12] The OD_{450nm} for blank wells containing BSS alone were subtracted from the sample OD_{450nm}, and the percentage peroxidase release was calculated by the formula:

(OD_{450nm} of test samples/OD_{450nm} of lysed cells) x 100 = % release. The spontaneous release from negative controls was subtracted from all samples to give percent specific release. MMP-9 and LTF were determined by ELISA kits obtained from R&D Systems and Oxis Research Ltd. (Portland, OR, USA), respectively. EDN concentration was determined by radioimmunoassay in the laboratory of Dr. H. Kita (Mayo Clinic, Rochester, MN) as previously described.[28] For all mediators examined, results (in triplicate) from separate experiments were expressed as mean percent release of control according to the formula: $[(OD_{test\ Ab} - OD_{neg\ control}) / (OD_{isotype\ Ab} - OD_{neg\ control})]$ x 100. Values from separate experiments were averaged and shown with error bars representing SEM.

M. FACS analysis of streptolysin-*O* (SLO)-permeabilized cells: entry of exogenous antibodies

It was previously demonstrated by Lindau and Gomperts (1991)[23] that exogenously added lactate dehydrogenase (LDH) could rapidly enter mast cells following SLO-permeabilization. To demonstrate that antibodies could similarly enter eosinophils and PMNs with rapid kinetics, we examined the uptake of exogenously added fluorescently-labeled antibodies following SLO permeabilization. Cells were washed 3 times in buffered saline solution (BSS) (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 20 mM PIPES, pH=6.8), resuspended at 1 x 10^6 cells/ml and incubated with 1.0 μg/ml phycoerytherin (PE)-conjugated anti-mouse antibodies (Cedarlane) in the presence or absence of 1.0 μg/ml (eosinophils) or 0.1 μg/ml (neutrophils) SLO. Samples were warmed for 1 min at 37°C to initiate permeabilization and examined

continuously by flow cytometry for the change in fluorescence intensity for 8.5 min. Results from this experiment (Figure 2.3, *page 112*) demonstrated that antibodies rapidly entered (within 1 min) SLO-permeabilized eosinophils and neutrophils.

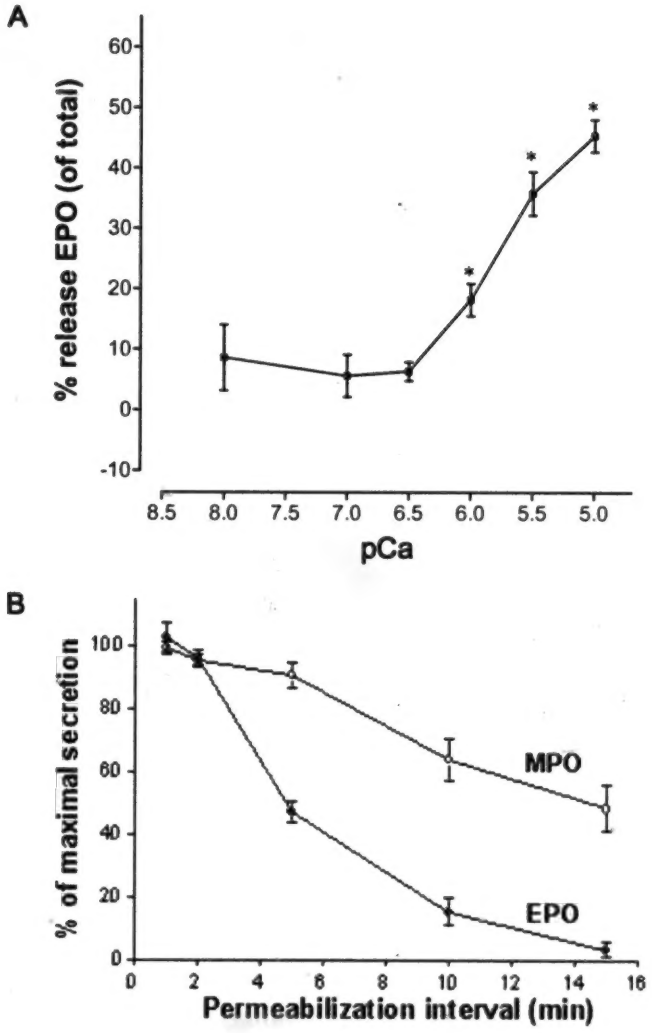


Figure 2.2

Secretory responsiveness to elevation of calcium and following streptolysin-*O* (SLO) permeabilization (A) Eosinophils were permeabilized in 1.0 mg/ml SLO containing different doses of calcium (pCa7-pCa5) supplemented with 10 mM GTPgS. Negative permeabilized controls (pCa8) were incubated in the absence of GTPgS. Cell supernatants were examined for EPO content following 10 min incubation at 37 °C, and % total release calculated as described in the Materials and Methods.(*) $p<0.05$ from negative control, as determined by the paired T test (n=5) **(B)** Exocytosis of EPO and MPO was determined for eosinophils and neutrophils, respectively, after increasing periods of permeabilization with SLO (permeabilization interval) prior to activation with Ca^{2+} (pCa 5) and 10 mM GTPgS. Values are expressed as percentage of maximal release which was detected at the time = 0 permeabilization interval (58% ± 14% EPO and 68% ± 8% MPO of total cellular peroxidase activity).

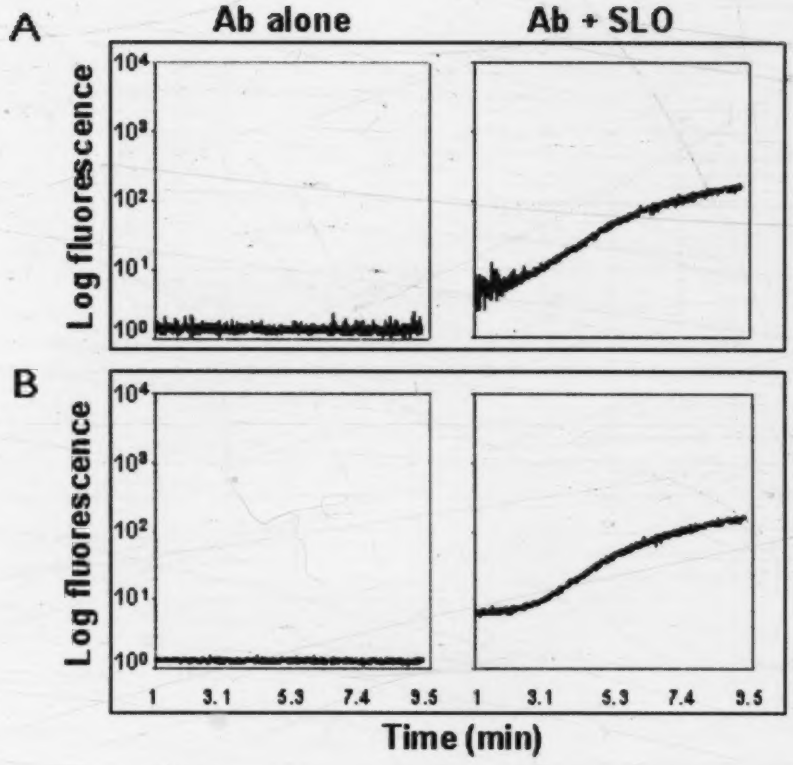


Figure 2.3
Entry of exogenous antibodies into SLO permeabilized granulocytes. Eosinophils (A) and neutrophils (B) were incubated with 1.0 mg/ml phycoerythrin (PE)-labeled antibodies in the absence or presence of 1.0 mg/ml or 0.1 ug/ml SLO, respectively, for 1 min at 37°C. Cells were then examined continuously by flow cytometery for the change in log fluorescence intensity for ~8.5 min.

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Chapter 3

The fusion protein vesicle-associated membrane protein-2 is implicated in IFNγ-induced piecemeal degranulation in human eosinophils from atopic individuals

Paige Lacy, PhD, Michael R. Logan, BSc, Ben Bablitz, BSc, MLT, and Redwan Moqbel, PhD, FRCPath

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M. Logan contributed to data generated in this manuscript as follows:

- *Western blot analysis of VAMP-2*
- *Tetanus toxin (TeNT) digestion of VAMP-2 in light membrane fractions*
- *Confocal microscopy of RANTES and VAMP-2 (IFN-γ time course): n=1*
- *Wrote methods and results section for VAMP-2 Western blot and tetanus toxin digestion experiments*

Abstract

Background: Exocytosis is an integral event during IFNγ-induced piecemeal degranulation in eosinophils. In many tissues, SNAREs (SNAP receptors), including vesicle-associated membrane protein (VAMP), act as specific intracellular receptors to allow granule fusion with the membrane during degranulation. However, the mechanisms underlying eosinophil piecemeal degranulation induced by IFNγ are not well understood. **Objective:** To assess whether eosinophils express the vesicular SNARE protein VAMP-2, and to determine the involvement of VAMP-2 in IFNγ-induced piecemeal degranulation. **Methods:** Human peripheral blood eosinophils (≥97%) from atopic subjects were subjected to RT-PCR and sequence analysis using specific primers for VAMP-2 mRNA. Western blot and flow cytometry analysis was carried out to confirm the identity of VAMP-2 and its susceptibility to cleavage by tetanus toxin. Confocal laser scanning microscopy imaging was conducted on double-labeled cytospins of eosinophils at 0, 5, 10, 30, 60 min and 16 h of IFNγ (500 U/ml) stimulation. **Results:** Eosinophils expressed VAMP-2 mRNA (n = 4 donors) which exhibited 100% homology with human VAMP-2 cDNA upon sequencing. Eosinophils were also found to express tetanus toxin-sensitive VAMP-2 protein. RANTES and VAMP-2 immunofluorescence were observed to colocalize to similar intracellular structures by confocal imaging. IFNγ induced a rapid translocation of VAMP-2-positive organelles towards the cell membrane in correlation with RANTES. **Conclusions:** These findings suggest that exocytosis in human eosinophils is regulated by SNAREs, with a specific role indicated for VAMP-2 in piecemeal degranulation.

Introduction

SNARE proteins have been implicated as critical regulators of membrane fusion at all levels of membrane trafficking in eukaryotic cells. These integral membrane molecules were first described for their ability to bind *N*-ethylmaleimide-sensitive factor (NSF) and soluble NSF-attachment proteins (SNAPs).[1] Their role in regulation of exocytosis in neuronal tissues is highlighted by the ability of tetanus and botulinum neurotoxins to cleave these molecules *in situ* and prevent further release of vesicular contents.[2] The paradigm of SNARE function proposes that binding of vesicular SNAREs (v-SNAREs) to cognate intracellular receptors on target membranes (t-SNAREs) must occur in order to drive membrane fusion.[1;3] The specificity of donor-target membrane fusion is postulated to be dependent on SNARE isoforms specific to each membrane compartment. Several isoforms of v-SNAREs have been identified, including vesicle-associated membrane protein (VAMP/synaptobrevin).[4-6] In the case of exocytotic release, VAMP-1 and VAMP-2 have been implicated as granule-associated v-SNAREs which recognize and bind to t-SNAREs in the plasma membrane.[7] More recent studies have demonstrated that cells outside of the neuronal/neuroendocrine system also express VAMP-1 and VAMP-2.[8-10]

Eosinophils are specialized secretory cells which have been shown to release some or all of the contents of their crystalloid granules following recruitment and activation in allergic inflammation and contribute to mucosal damage.[11] The process of eosinophil granule mediator release in tissues appears to occur by either compound exocytosis,[12] piecemeal degranulation,[13] or necrotic release.[14] Piecemeal degranulation seems to be the most physiologically relevant form of eosinophil

degranulation in allergic inflammation, since approximately 67% of all tissue eosinophils observed in nasal biopsies from individuals with seasonal allergic rhinitis appear to undergo piecemeal degranulation, with the remainder exhibiting necrotic or cytolytic release.[14] *In vitro* studies on peripheral blood eosinophils have shown that IFNγ selectively induced the release of a chemokine, RANTES,[15] in a manner closely resembling piecemeal degranulation observed *in vivo*. However, the mechanisms underlying this important physiological process are not yet understood. In an earlier study, a number of isoforms of SNARE proteins, including VAMP-1, could not be detected in guinea pig eosinophils (VAMP-1, VAMP-3/cellubrevin, synaptotagmin, synaptophysin, syntaxin-1 and SNAP-25).[16] We hypothesized that, instead, human eosinophils express VAMP-2 mRNA and protein, and that VAMP-2 is rapidly mobilized together with RANTES during IFNγ-induced piecemeal degranulation. These observations show for the first time that the SNARE isoform, VAMP-2, may be involved in the regulation of eosinophil secretory processes.

Results

VAMP-2 mRNA is expressed in eosinophils. Purified eosinophils were subjected to RT-PCR analysis using a previously published set of primers specific for VAMP-2.[17] Eosinophils from four different atopic subjects demonstrated VAMP-2 mRNA expression (Fig. 3.1 A). Eosinophils from one normal nonatopic subject were also found to express VAMP-2 mRNA (data not shown), suggesting that the gene encoding VAMP-2 may be expressed regardless of disease or activation status. Eight clones were prepared from a PCR product obtained from a representative atopic subject. Four clones were subjected to sequence analysis by the dideoxy chain-termination method. Eosinophil mRNA for VAMP-2, containing the entire coding sequence of the molecule (348 bp), exhibited 100% homology to that of human cDNA encoding VAMP-2 (NIH Genbank accession no. NM_014232.1). The predicted amino acid sequence derived from eosinophils was inclusive of all known toxin cleavage sites for VAMP-2 (tetanus toxin, botulinum toxins B, D, F, and G).

Eosinophils express VAMP-2 immunoreactivity sensitive to cleavage by tetanus toxin. Eosinophil membranes were prepared and subjected to incubation with reduced tetanus toxin at 37°C before separation by SDS-PAGE and immunoblot analysis using monoclonal antibody to VAMP-2. The light membranes prepared from blood eosinophils exhibited immunoreactivity for VAMP-2 migrating to 18 kDa, equivalent to that found in rat brain preparations, which was sensitive to cleavage by reduced tetanus toxin but not boiled (inactive) toxin (Fig. 3.1 B). Immunoblot analysis with an isotype control (mouse IgG_1), at a similar concentration to anti-VAMP-2, did not yield any immunoreactive bands (data not shown).

Eosinophils express VAMP-2 protein at lower levels than the crystalloid granule marker CD63. Expression of VAMP-2 protein in eosinophils was determined by flow cytometry analysis of formaldehyde-fixed and permeabilized eosinophils. Eosinophils were analyzed for intracellular VAMP-2 immunoreactivity in comparison with a crystalloid granule membrane marker, CD63.[18;19] Permeabilized eosinophils demonstrated minimal labeling with isotype control (Fig. 3.2 A) while CD63 staining was significantly greater than control (16-fold increase in MFI over isotype; see Table below Fig. 3.2 A). Conversely, cells stained with anti-VAMP-2 showed a smaller, although still significant, increase in immunofluorescence, with a 1.75-fold increase in MFI over isotype control ($p < 0.001$).

$CD63^+$ crystalloid granules do not express VAMP-2. The expression of VAMP-2 protein on isolated, purified eosinophil crystalloid granules was determined by flow cytometry analysis. Interestingly, while CD63 expression was detectable in a population of gated crystalloid granules (23-fold increase in fluorescence over isotype control; $p < 0.001$), VAMP-2 was not significantly expressed in this population (Fig. 3.2 B).

VAMP-2 was rapidly mobilized during IFNγ-induced piecemeal degranulation. Double-labeling of eosinophil cytospins was carried out in order to examine the expression of VAMP-2 in comparison with an eosinophil-derived mediator, RANTES, known to be stored in both the crystalloid granules and a putative population of small secretory vesicles.[15]

In eosinophils obtained from four atopic donors, VAMP-2 co-localized with RANTES in what appeared to be small cytoplasmic bodies, which appear yellow due to coinciding green and red fluorescence (Fig. 3.3 B and D). In addition, eosinophil

VAMP-2 and RANTES co-localized with each other in perinuclear regions, which were also positive for calnexin immunoreactivity (Fig. 3.3 L). The overlap in staining of VAMP-2 and RANTES with that of calnexin suggests some expression of these molecules in the endoplasmic reticulum.

In a time course analysis of eosinophil cytospins prepared at 0, 2, 5, 10, 30, 60 min, and 16 h of stimulation, VAMP-2 and RANTES immunoreactivity were mobilized together to the plasma membrane during IFNγ-induced (500 U/mL) piecemeal degranulation (Fig. 3.3 C, *arrow*, and G). After as early as 5 min, VAMP-2 immunoreactivity was intensified around the periphery of stimulated cells in a highly punctate manner (Fig. 3.3 F, *arrowhead*). IFNγ also induced the redistribution of RANTES immunoreactivity after 10 min as previously reported,[15] with some loss of RANTES activity in cytoplasmic regions accompanied by an intensification in the submembranous regions of the cell (Fig. 3.3 C and G). Figure 3.3 B and C represent cells obtained from a different donor from that of Fig. 3 .3 D-J, in which a particularly pronounced translocation of RANTES and VAMP-2 could be observed (Fig. 3.3 C, compared with unstimulated cells in Fig. 3.3 B). In this example, RANTES and VAMP-2 could be observed to co-localize strongly in tightly punctate structures immediately adjacent to the cell membrane, suggesting that exocytotic release of RANTES may be occurring. The more commonly observed time course of redistribution of immunoreactivity for VAMP-2 and RANTES in most cells responding to IFNγ is shown in Fig. 3.3 D-J.

The redistribution of cytoplasmic immunoreactivity for VAMP-2 and RANTES in double-labeled IFNγ-treated cells was found to be statistically significant (Fig. 3.4 A).

RANTES immunoreactivity was significantly reduced ($p < 0.001$) at 5, 10, 30, and 60 min post IFNγ stimulation. The immunofluorescence for RANTES returned to basal levels after 16 h of continuous incubation with IFNγ. MBP immunoreactivity was also depleted significantly after 5 and 30 min ($p < 0.01$) of stimulation by IFNγ compared with control (t = 0) values (Fig. 3.4 A). While VAMP-2 immunoreactivity was not significantly changed from control (t = 0) values throughout the time course, it was significantly decreased at times 5, 10, and 30 min compared with 2 min measurements (Fig. 3.4 A) in parallel with values obtained for RANTES. Interestingly, cell profiles of pixel intensities from representative cells taken from 0 and 10 min of IFNγ stimulation (Fig. 3.4 B) exhibited a pronounced shift in VAMP-2 and RANTES immunoreactivity towards the cell membrane (bold lines), as well as a significant depletion in cytoplasmic RANTES. These observations indicate that, along with depletion in cytoplasmic immunoreactivity for VAMP-2 and RANTES, these molecules were mobilized together to the cell membrane. RANTES immunoreactivity has previously been shown to be detected in supernatants of IFNγ-stimulated eosinophils after 60-120 min of incubation, suggesting release of RANTES during degranulation concurrently with intracellular translocation of VAMP-2 and RANTES.[15]

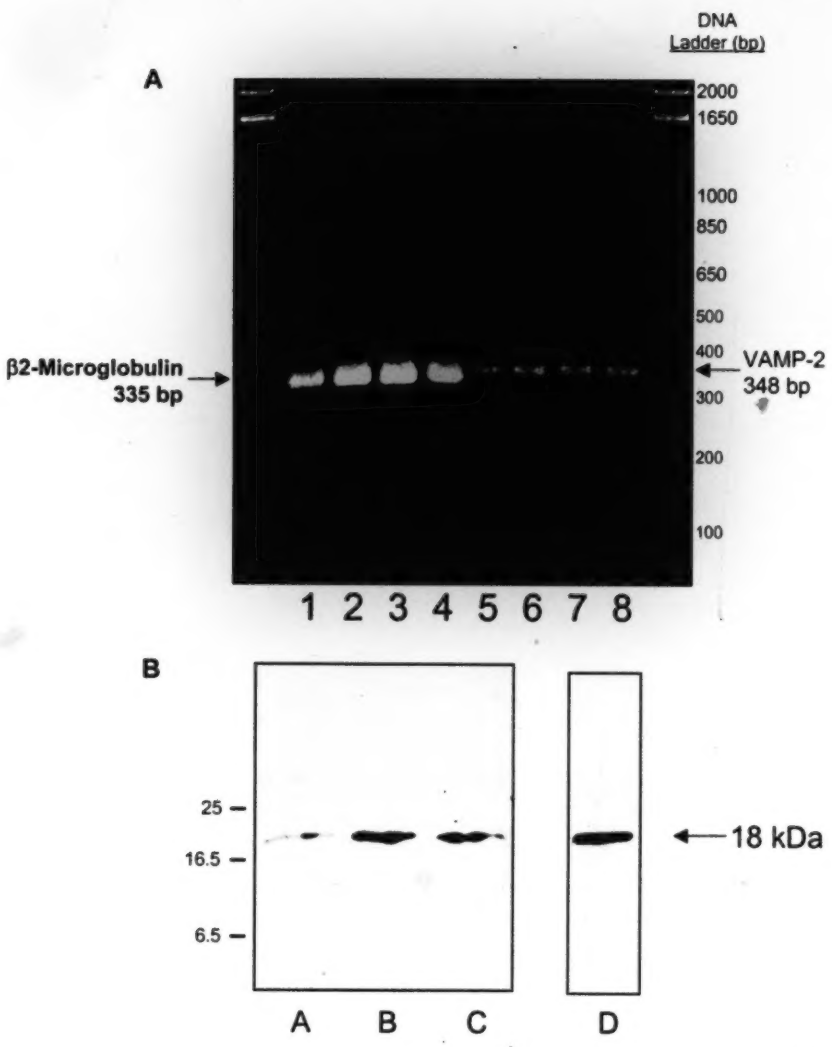
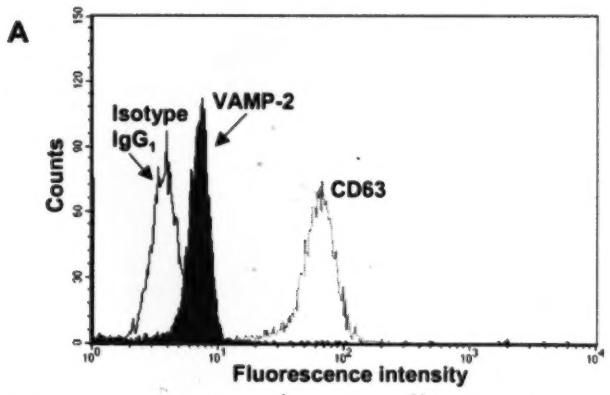
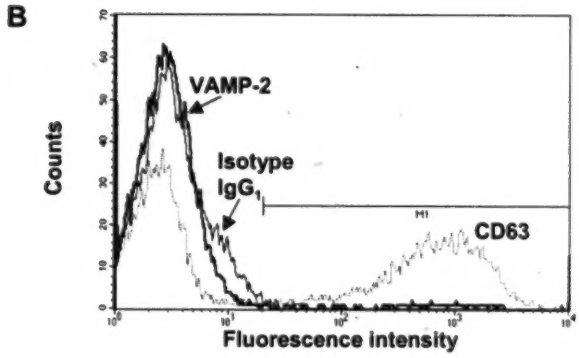


Figure 3.1. VAMP-2 expression in human eosinophils from atopic subjects. (A) Products of RT-PCR (2 x 10^6 cells/sample) were visualized on an ethidium bromide-stained 2% agarose gel. Lanes 1-4 represent PCR products for β2-microglobulin (335 bp) obtained from eosinophil mRNA purified from four different atopic donors. Lanes 5-8 represent VAMP-2 PCR products (348 bp) obtained from the same group of atopic donors. Lanes are flanked by DNA ladders. (B) Immunoblot analysis of VAMP-2 in eosinophils, following incubation with reduced tetanus toxin (lane A), boiled tetanus toxin (lane B), and in the absence of toxin (lane C). Lanes A-C each contain membranes prepared from 5 x 10^6 cell equivalents. Eosinophil light membranes were also titrated (lane D, 10 x 10^6 cell equiv., lane E, 5 x 10^6 cell equiv., lane F, 2.5 x 10^6 cell equiv., lane G, 1 x 10^6 cell equiv.) and electrophoresed concurrently with rat brain positive control (lane H, 0.5 μg rat brain synaptosomes. Numbers at left indicate the positions of prestained molecular weight markers. (Figures 3A and 3B were generated by P.Lacy and M. Logan, respectively.)



Label	MFI	SEM
Isotype (IgG_1)	3.88	0.01
VAMP-2	6.79***	0.14
CD63	63.57***	1.48



Label	% gated (M1)	SEM
Isotype (IgG_1)	1.98	0.15
VAMP-2	2.86	0.50
CD63	46.97***	0.84

Figure 3.2. Flow cytometry analysis of VAMP-2 expression in permeabilized eosinophils and purified crystalloid granules. (A) Permeabilized eosinophils stained with isotype control antibody (mouse IgG_1; solid line), VAMP-2 antibody (solid area), and CD63 antibody (dotted line). The average ± SEM values of MFI is shown in the table beneath the figure from 10,000 events per measurement. (B) Crystalloid granules obtained from density gradient centrifugation were fixed (without permeabilization) and labeled with isotype control (light solid line), VAMP-2 antibody (heavy solid line), and CD63 antibody (dotted line). The average ± SEM percentage of gated events (M1; gated for $CD63^+$ granules) is shown beneath the diagram from 10,000 events per measurement. Results are averaged from three measurements taken from a representative sample. *** indicates $p < 0.001$.(Figure 3.2 was generated by P. Lacy)

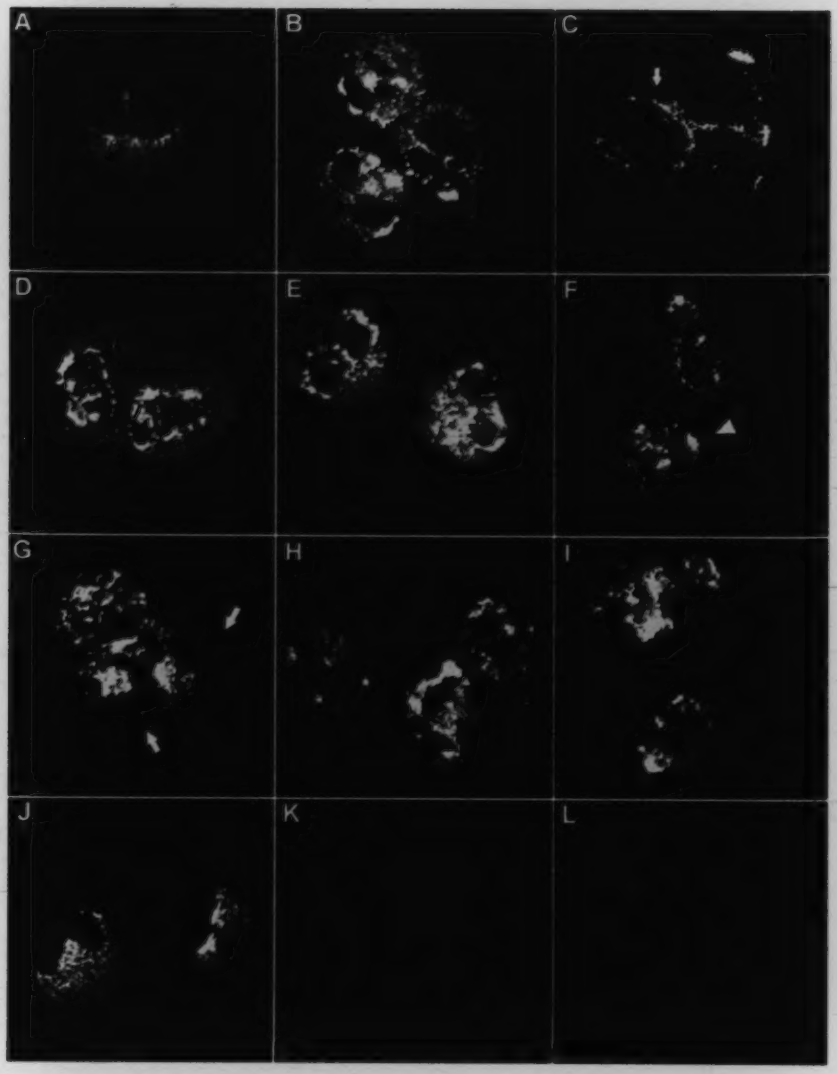


Figure 3.3. Confocal microscopy analysis of double-labeled eosinophils following stimulation with IFNγ. (A) Immunofluorescence detection of VAMP-2 (green color from BODIPY FL) in an eosinophil, which co-localized with RANTES (red color from TRITC) to produce yellow regions in (B) and (D). IFNγ was added to cells (500 U/mL) and the distribution of VAMP-2 and RANTES was analyzed following 0 min (D), 2 min (E), 5 min (F), 10 min (G), 30 min (H), 60 min (I), and 16 h (J) at 37°C. (K) Negative control with an isotype antibody (mouse IgG_1). (L) Calnexin stain indicating endoplasmic reticulum distribution in eosinophils. (F) *Arrowhead*, punctate staining of VAMP-2 immunofluorescence observed at 5 min of stimulation, followed by translocation of VAMP-2 and RANTES (*arrows* in C and G). Orig. mag, 100X. (Figure 3.3 was generated by P. Lacy (n=2) and M. Logan (n=1))

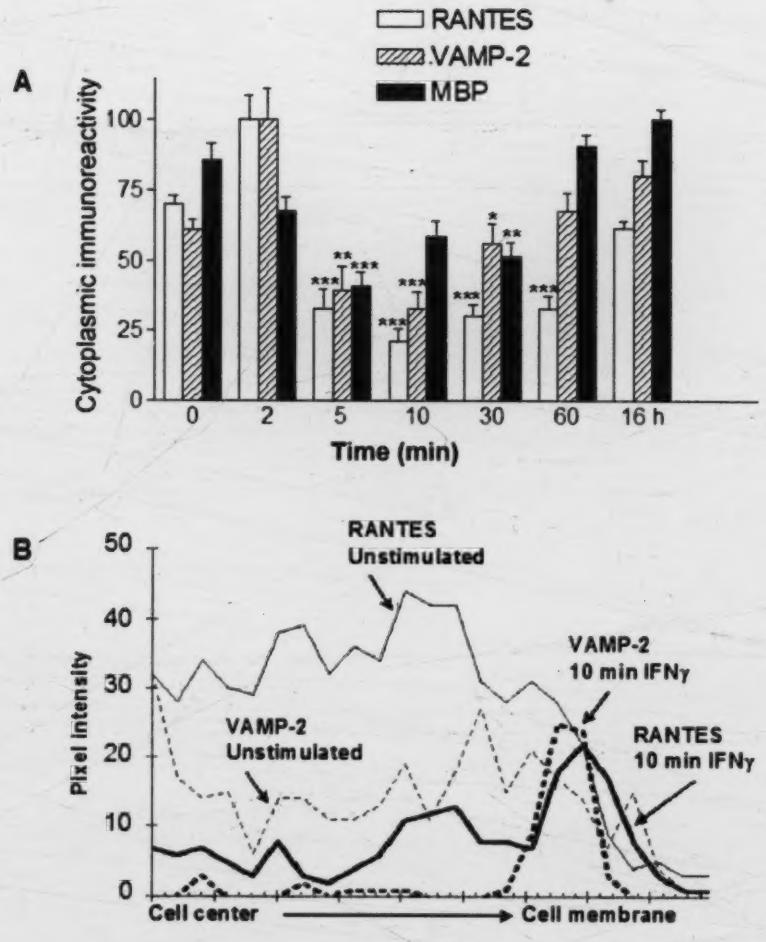


Figure 3.4. RANTES mobilization from IFNγ-stimulated eosinophils occurs concurrently with translocation of VAMP-2. (A) Intensity of cytoplasmic immunofluorescence for RANTES, VAMP-2, and granule MBP following IFNγ stimulation. Values were obtained from the same population of cells shown in Fig. 3 (D-J). Statistical comparisons for RANTES and MBP were carried out using t = 0 as the control (t = 2 min for VAMP-2). (B) Cell profiles of immunofluorescence along the radii of representative cells before (light lines) and after 10 min IFNγ stimulation (heavy lines). RANTES immunoreactivity is indicated by solid lines, while VAMP-2 is shown in dotted lines. * indicates $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ using one-way analysis of variance (Kruskal-Wallis) followed by Dunn's multiple comparison test. (Figure 3.4 was generated by P. Lacy)

Discussion

We report here that human eosinophils express VAMP-2 mRNA and protein, which was sensitive to cleavage by tetanus toxin. Furthermore, we demonstrate that eosinophil VAMP-2 co-localized with RANTES, which were both rapidly mobilized towards the plasma membrane in response to IFNγ stimulation in advance of RANTES release. VAMP-2 is known to be essential for, or at least implicated in, the process of exocytotic secretion in many cell types.[8;20-22] We now propose that piecemeal degranulation, a major physiological event in eosinophilic inflammation, may be regulated by VAMP-2.

The nucleotide sequence obtained from cloning of the eosinophil VAMP-2 PCR product showed an exact match with that of human VAMP-2, suggesting that eosinophils employ an identical molecule for regulation of exocytosis as described in other tissues.[1;6] Interestingly, the expression pattern of VAMP-2 appeared to be restricted to $CD63^-$ secretory vesicles. Immunoreactivity for VAMP-2 co-localized with RANTES, suggesting that it may belong to a pool of rapidly mobilizable small secretory vesicles sensitive to IFNγ stimulation previously described.[15] We now have compelling evidence for a role for VAMP-2 in mediating selective release of RANTES from eosinophils, a process akin to piecemeal degranulation described in other studies.[14;15] The peripheral redistribution of the VAMP-2 immunofluorescence was accompanied by a depletion of signal from the cytoplasm, suggesting that VAMP-2-containing small secretory vesicles had mobilized towards the plasma membrane. In addition, the antibody to VAMP-2 used in this study recognizes the N-terminus sequence of the molecule, which is occluded during trans-SNARE complex formation.[23] Our finding

that VAMP-2 immunoreactivity increased slightly, but not significantly, after 2 min of IFNγ stimulation suggests that a few of the VAMP-2 molecules may have been partially occluded in resting or unstimulated cells.

We were unable to test the functional effect of tetanus toxin on IFNγ-induced degranulation for the reason that this requires permeabilization of the cell membrane in order to allow the toxin light chain (its catalytic component) into the cytoplasm. Non-neuronal cells do not exhibit any capacity for binding and endocytosis of clostridial toxins, which is the postulated mechanism of action in neuronal cells.[24] Permeabilization techniques (using streptolysin-O, for example) render receptor-coupled mechanisms refractory to further stimulation due to washout of cytosolic signalling molecules.[25] Thus, permeabilization of eosinophils followed by IFNγ stimulation is not feasible, since intracellular signalling pathways are likely to be severely interrupted by cytosolic washout of proteins. Other strategies would have to be employed to directly demonstrate an involvement of VAMP-2 in IFNγ-induced eosinophil degranulation.

Eosinophil VAMP-2 expressed on secretory vesicles is likely to bind to cognate t-SNAREs in the plasma membrane during the process of exocytosis. We have recently identified the expression of the t-SNARE isoforms SNAP-23 and syntaxin-4 in human eosinophils, which appear to be localized predominantly in the plasma membrane. Further studies will be carried out to investigate the functional interaction of these SNARE molecules.

The intriguing finding that eosinophil crystalloid granules were negative for VAMP-2 immunoreactivity suggests that VAMP-2 may not regulate membrane fusion of these granules with the plasma membrane. According to the SNARE hypothesis, in

order for membrane trafficking to occur between granules or other membrane-bound compartments, there must exist a set of intracellular membrane receptors that recognize and bind each other to enable specific fusion. In light of these observations, a new, distinct and as yet unidentified isoform of VAMP may exist on the membranes of crystalloid granules to facilitate the shuttling of small vesicles as part of piecemeal degranulation or to fuse directly with the plasma membrane during compound exocytosis.

In summary, we propose that eosinophils express VAMP-2, which we believe is critical for controlling the exocytotic machinery in these cells. A role for VAMP-2 in eosinophil secretion may be particularly relevant in processes involving piecemeal degranulation. The SNARE molecules may lend themselves to immunopharmacological manipulation. Modulation or prevention of SNARE-regulated degranulation in eosinophils may prevent damaging sequelae associated with asthmatic inflammation.

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Chapter 4

Expression of eosinophil target SNAREs as potential cognate receptors for VAMP-2 in exocytosis

Michael R. Logan*, BSc, Paige Lacy*, PhD, Ben Bablitz, BSc, and Redwan Moqbel, PhD, FRCPath

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Abstract

Exocytosis of eosinophil granule-derived mediators is thought to be an important effector response contributing to allergic inflammation. Secretion from many cell types has been shown to be dependent on the formation of a docking complex composed of SNAREs (SNAP receptors) located on the vesicle (v-SNAREs) and the target membrane (t-SNAREs). SNARE isoforms, VAMP-2, SNAP-23 and syntaxin-4, have been described in secretory processes in myeloid cells. Previously, we have demonstrated that the v-SNARE, VAMP-2, is a candidate v-SNARE involved in eosinophil exocytosis, and is localized to a pool of $RANTES^{+}$ vesicles that translocate to the cell periphery after IFN-γ-induced degranulation. The objective of this study was to determine whether eosinophils express the t-SNARE isoforms, SNAP-23 and syntaxin-4, as potential binding targets for VAMP-2 during exocytosis. Human peripheral blood eosinophils (>97%) from atopic subjects were subjected to RT-PCR and sequence analysis using specific primers for SNAP-23 and syntaxin-4. Protein expression and localization was determined by Western blot analysis of eosinophil subcellular fractions, and confirmed by confocal laser scanning microscopy (CLSM). Nucleotide sequences obtained from PCR products exhibited nearly identical (>95%) homology with reported sequences for human SNAP-23 and syntaxin-4. Both SNAP-23 and syntaxin-4 were present in plasma membranes, with some staining in ER and Golgi. Negligible expression was detected in crystalloid and small/secretory granules. Plasma membrane-associated t-SNAREs, SNAP-23 and syntaxin-4, are expressed in human eosinophils, and are likely candidates for association with VAMP-2 during docking which is followed by exocytosis.

Introduction

Eosinophils are prominent inflammatory cells with the potential to act in an immunomodulatory manner in allergic inflammation.[1] Piecemeal degranulation (PMD) has been identified as a major mechanism for exocytotic release intissue eosinophils obtained from patients with allergic rhinitis. [2] PMD has also been observed to occur *in vitro* following physiological stimulation.[3-6]

Degranulation is dependent on a membrane fusion event (exocytosis) in which SNARE fusion proteins are thought to play a crucial role.[7;8] Exocytosis occurs following the formation of a ternary SNARE complex consisting of the vesicular SNARE (v-SNARE), VAMP (vesicle-associated membrane protein), and two target membrane SNAREs (t-SNAREs), syntaxin and SNAP-25 (synaptosome-associated-protein of 25 KDa).[9] Isoforms of SNAREs have been described in non-neuronal tissues, and have a critical role in exocytosis.[7;8;10-13]

We have established an *in vitro* model to study PMD in eosinophils which involves the selective mobilization and release of RANTES following stimulation with IFN-γ.[3] In a recent report, we demonstrated that human eosinophils express the v-SNARE VAMP-2, which localized to RANTES$^+$ secretory vesicles distinct from crystalloid granules. VAMP-2 translocation was induced upon cell activation with IFN-γ, and coincided with RANTES mobilization to the plasma membrane.[14] The presence of VAMP-2 in human eosinophils has also been reported in separate studies, and these findings may have important implications for a role for VAMP-2 in eosinophil mediator release.[15] VAMP-2 is capable of forming SNARE complexes with the t-SNAREs, SNAP-23 and syntaxin-4, *in vitro*.[16;17] Recent studies indicate

that these t-SNAREs may be critical components of the exocytotic machinery in mast cells, platelets and neutrophils.[7;12;13;18-20] The expression and localization of SNAP-23 and syntaxin-4 in human eosinophils has not been examined. We hypothesized that human eosinophils express syntaxin-4 and SNAP-23 which are localized to the cell membrane, serving as potential binding partners for VAMP-2 during PMD. In this report, we demonstrate for the first time that syntaxin-4 and SNAP-23 proteins are expressed in association with the plasma membrane of human eosinophils, and may be critical components for exocytosis.

Results

Syntaxin-4 and SNAP-23 mRNA are expressed in eosinophils. Purified eosinophils were subjected to RT-PCR analysis using specific primers as described. Eosinophils from four different atopic subjects exhibited mRNA expression for both SNAP-23 (498 bp) and syntaxin-4 (894 bp) (Fig 4.1). Consistent with the previous report, we also detected a faint band at 340 bp, which may indicate expression of a splice variant, SNAP-23B [21] (Fig 4.1A). Eosinophils from a non-atopic donor were also found to express both SNAP-23 and syntaxin-4 mRNA (data not shown), indicating that gene expression of these two proteins is not exclusive to allergic disease.

Sequences for both SNAP-23 and syntaxin-4 coding regions were obtained from eosinophils of a representative atopic donor. Sequencing of eosinophil cDNA derived from SNAP-23-specific primers exhibited 97% homology to that of human cDNA for SNAP-23A (NIH Genbank Access. No. U55936). The PCR product generated from eosinophil cDNA for syntaxin-4 also exhibited 97% homology to the

reported human cDNA sequence for syntaxin-4 (NIH Genbank Access. No. AF026007).

SNAP-23 and syntaxin-4 are expressed in eosinophil light membrane fractions. Eosinophil light membranes were prepared and subjected to separation by SDS-PAGE and immunoblot analysis, using either anti-SNAP-23 or anti-syntaxin-4. Platelet lysate (10 μg protein/lane) was used as a positive control for SNAP-23 and syntaxin-4 protein expression.[13;20;22] Light membranes from eosinophils (enriched in small secretory vesicles, plasma membranes and Golgi membranes, but devoid of nuclei and crystalloid granules) exhibited immunoreactivity for both SNAP-23 and syntaxin-4, which comigrated with the positive control, to apparent molecular weights of approximately 30 kDa and 35 kDa, respectively (Fig 4.2A & B). No immunoreactivity was obtained using either of the control antibodies, normal rabbit serum and mouse IgG_1 (data not shown).

SNAP-23 and syntaxin-4 are localized to eosinophil plasma membranes and intracellular regions. Consistent with previous findings, organelles were separated to different density regions of the Nycodenz gradient as determined by peak activities of marker proteins (Fig 4.3A).[3;23] Subcellular fractions (30 μl) (0-7.5 μg protein/lane) were subjected to separation by SDS-PAGE and immunoblot analysis using either anti-SNAP-23 or anti-syntaxin-4. Optical densities of immunoreactive bands for SNAP-23 (Fig 4.3B) and syntaxin-4 (Fig 4.3C) revealed near identical distribution in the gradient, each characterized by a major peak in activity with a shoulder of immunoreactivity in higher density fractions. Peak optical density of both SNAP-23 and syntaxin-4 precisely coincided with plasma membrane-rich fractions (Fig 4.3A).

Both SNAP-23 and syntaxin-4 were not detected in EPO or β-Hex-positive fractions, indicating that crystalloid granules do not express these t-SNAREs. SNAP-23 immunoreactivity could be faintly detected in the LDH-containing cytosolic fraction, which may be the result of homogenization causing disruption and detachment of SNAP-23, a peripheral membrane protein.

Plasma membrane and other intracellular sites of expression of SNAP-23 and syntaxin-4 was confirmed by CLSM. Eosinophil cytospins were prepared and immunolabeled for syntaxin-4 (n=3) or SNAP-23 (n=3). In both cases, minimal labeling was observed for isotype control antibodies compared to detectable fluorescence using SNARE-specific antibodies (Fig 4.4). Specific labeling of both SNAP-23 and syntaxin-4 was detected at the cell periphery, which confirmed localization to the plasma membrane observed in our analysis of subcellular fractions. A highly focused perinuclear region exhibited intense immunoreactivity for SNAP-23, closely resembling the staining pattern obtained with anti-giantin, a *cis*-Golgi membrane marker,[24;25] (Fig 4.4B). To a lesser extent, punctate cytoplasmic staining for SNAP-23 was also detected. Perinuclear immunoreactivity for syntaxin-4 was similarly detected, but was considerably less intense than SNAP-23 (Fig 4.4C) .

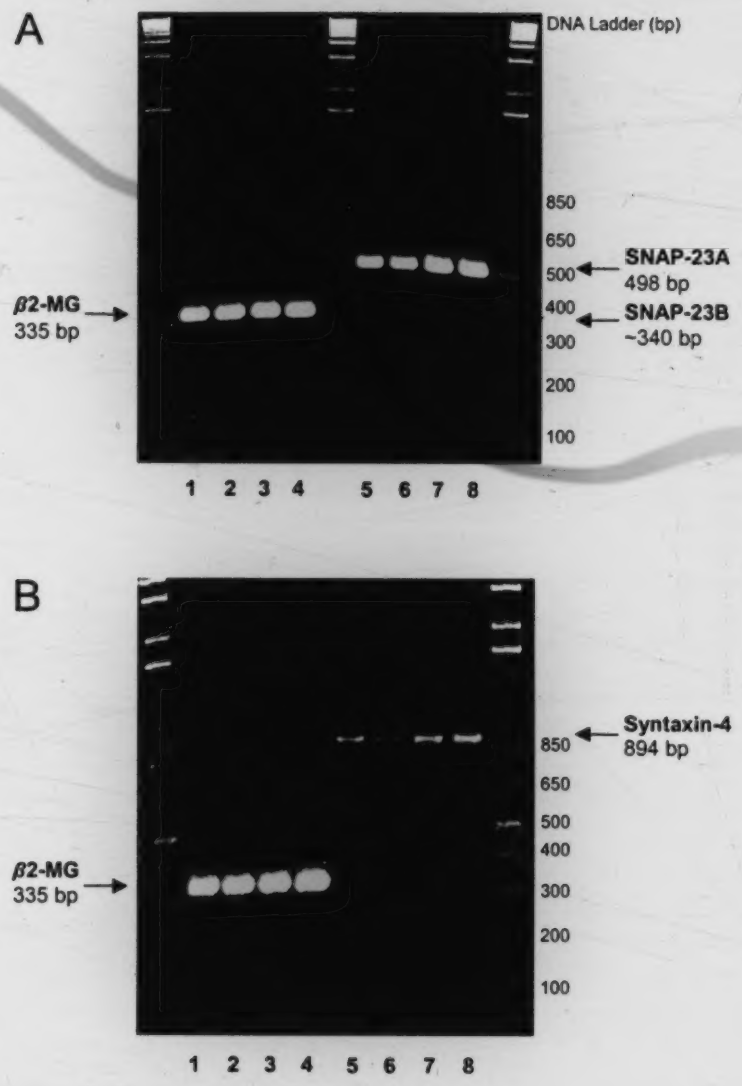

Figure 4.1. SNAP-23 and syntaxin-4 mRNA expression in human eosinophils from atopic subjects. Total RNA (2 x 10^6 eosinophils) was obtained from four different atopic donors and reverse transcribed and amplified (30 cycles) prior to gel analysis. Products were visualized on ethidium bromide-stained 2% agarose gels. (A) PCR products corresponding to SNAP-23A (498 bp) and, to a much lesser extent, SNAP-23B (~ 340 bp) were detected (Lanes 5-8). (B) Syntaxin-4 PCR products (894 bp) are shown in lanes 5-8. Each gel shows PCR products for β2-microglobulin control (β2-MG) (335 bp) (lanes 1-4), and is flanked by DNA ladders with sizes indicated on the right. (Figure 4.1 was generated by A. Johri)

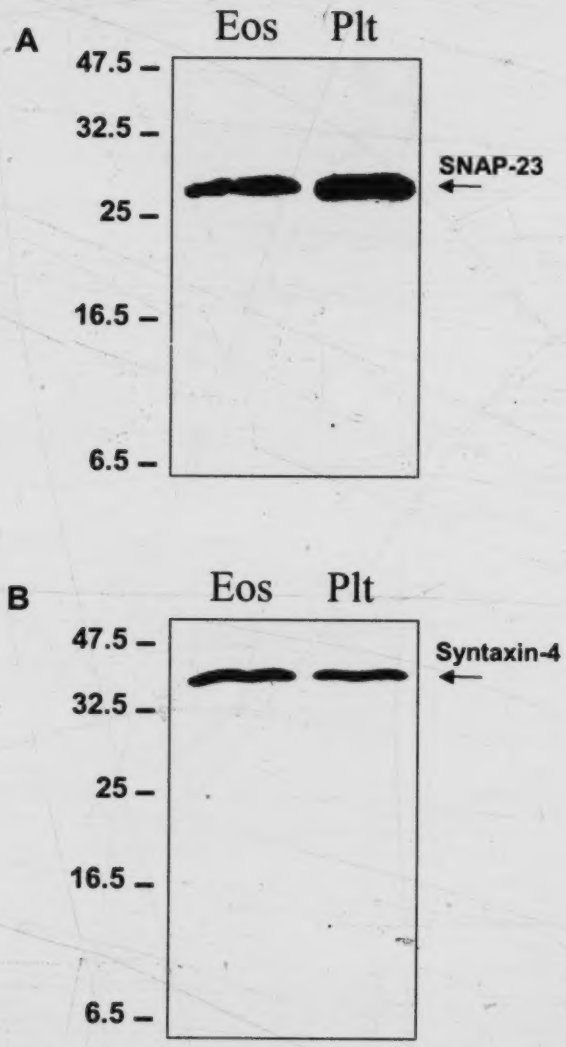

Figure 4.2. Eosinophils express SNAP-23 and syntaxin-4 in light membrane fractions. Eosinophil light membrane fractions were subjected to immunoblot analysis for SNAP-23 and syntaxin-4, as described in the Methods and Materials section. Shown here is a representative result from three separate experiments. Immunoreactivity of 5 x 10^6 cell equivalents of eosinophil light membrane fractions (Eos) to SNAP-23 (A) and syntaxin-4 (B) is shown in relation to concurrently electrophoresed 10 ug platelet lysate positive controls (Plt). Molecular weight markers are shown on the left. (Figure 4.2 was generated by M. Logan)

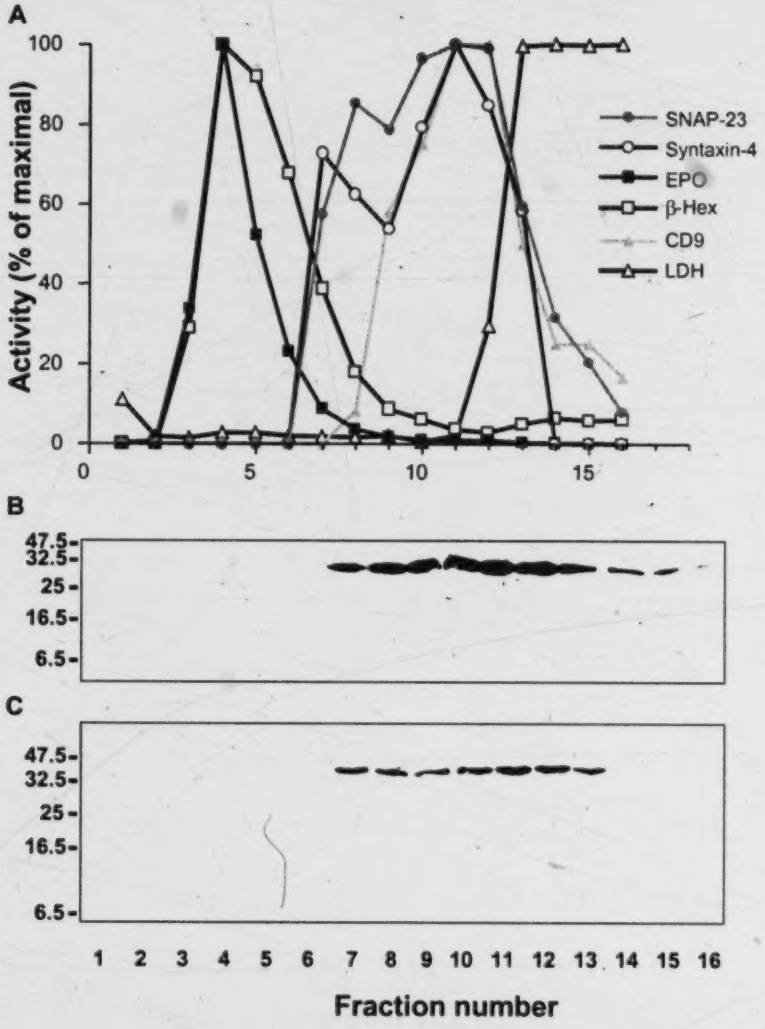

Figure 4.3. Subfractionation of eosinophils to show intracellular localization of SNAP-23 and syntaxin-4. (A) Subcellular distribution of intracellular compartments in density gradients prepared from eosinophils. Fractionated human peripheral blood eosinophils (5 x 10^7) were measured for EPO (crystalloid granules), β-hexosaminidase (crystalloid / small granules), CD9 (plasma membrane), and LDH (cytosol) to determine positions of respective intracellular compartments in the gradient. The activities of marker enzymes are depicted as percentages of maximal activity in fractions from the gradient. Fraction 1 contains 45% Nycodenz while fraction 13 contains 0% Nycodenz. Fractions 1-16 (0-7.5 μg protein/lane) were subjected to electrophoretic separation and immunoblot analysis for SNAP-23 (B) and syntaxin-4 (C). Molecular weight markers are shown on the left. Immunoreactive bands detected for SNAP-23 and syntaxin-4 were expressed as percentages of maximal optical density (A). Shown are representatives of two separate experiments. (Figure 4.3 was generated by P. Lacy (n=1) and M. Logan (n=2))

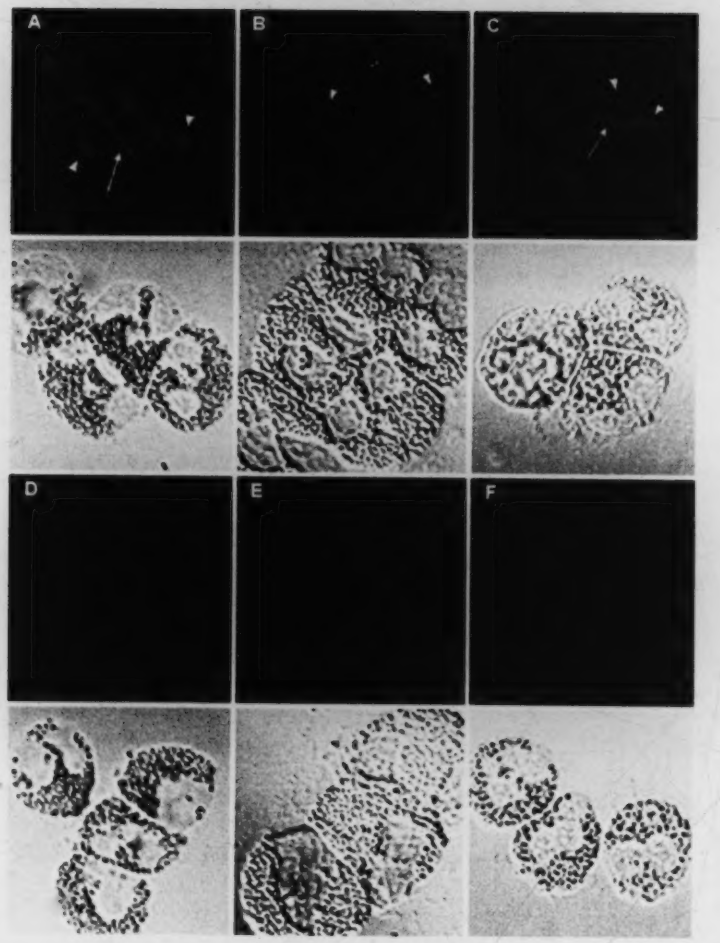

Figure 4.4. Immunofluorescent staining of eosinophils with SNAP-23 and syntaxin-4 antibodies. Plasma membrane (arrows) and perinuclear (arrowheads) immunofluorescence using specific antibodies on fixed, permeabilized eosinophil cytospins against SNAP-23 (A) and syntaxin-4 (C) are shown compared to isotype controls, affinity-purified rabbit IgG (D) and mouse IgG_1 (F), respectively. Golgi labeling (arrowheads) using a specific *cis*-Golgi marker, anti-giantin (B), is shown compared to its isotype control, normal rabbit serum (E). DIC microscopy images of cells are shown below in each case. Original magnification, 63x. (Figure 4.4 was generated by M. Logan)

Discussion

We report here that human eosinophils express the t-SNAREs, SNAP-23 and syntaxin-4, which may form an integral part of the cells' exocytotic machinery. In a previous study, we demonstrated that the v-SNARE VAMP-2 translocated in association with a $RANTES^{+}$ pool of small secretory vesicles, but not the crystalloid granules, to the plasma membrane during exocytosis.[14] In support of our findings, two parallel publications demonstrated that eosinophil immunoreactivity using antibodies specific for VAMP isoforms[15;17] was localized to small secretory vesicles, with minimal staining of crystalloid granules. From this, we sought to identify potential t-SNARE binding partners for VAMP-2 in human eosinophils.

Guinea pig eosinophils do not express the neuronal t-SNAREs, syntaxin-1 and SNAP-25,[26] which are the postulated binding partners for VAMP-2 in neuronal cells.[9] However, alternative t-SNARE isoforms, syntaxin-4[18-20;22;27] and SNAP-23[10], have been described in a wide variety of non-neuronal tissues, and appear to participate in important secretory roles during platelet[13;20] and mast cell[7;18] exocytosis. In support of our previous findings on VAMP-2, we propose that SNARE proteins may be critical components of the protein machinery required for eosinophil granule-derived mediator release during receptor-mediated exocytosis.

The nucleotide sequences obtained from cloning of SNAP-23 and syntaxin-4 PCR products were almost identical to their respective database sequences for human tissue homologs.[7;12;13] Subcellular fractionation analysis indicates that part of the intracellular expression of SNAP-23 and syntaxin-4 coincided with the plasma membrane, which was confirmed by CLSM. Plasma membrane staining for both

SNAP-23 and syntaxin-4 was more intense at regions of contact between cells, as compared to single cells (data not shown), which may be attributable to aggregation of antibody complexes. However, this is unlikely, since isotype control antibodies did not exhibit staining in similar cell aggregates, nor was any intensified membrane staining at points of cell contact found in *cis*-Golgi (anti-giantin) labeling. This suggests that eosinophils may employ these two t-SNAREs for regulation of exocytosis in a similar manner to other reported non-neuronal tissues.[28]

In addition to plasma membrane localization, we detected SNAP-23 immunoreactivity to a perinuclear region closely resembling *cis*-Golgi staining using anti-giantin. A previous report demonstrated similar labeling in HeLa cells co-transfected with SNAP-23 and syntaxin-11[29], and suggested that SNAP-23 may have a role in vesicle trafficking to late endosomes and/or the *trans*-Golgi network. However, it is possible this observation may have been the result of overexpression of SNAP-23 in these cells. We postulate that perinuclear staining for syntaxin-4 could equally reflect localization to the ER or transport vesicles. Consistent with this finding, previous reports indicate that syntaxin-4 expression is not exclusive to the plasma membrane. Syntaxin-4 is expressed on GLUT4-containing organelles in adipocytes[30], and may also be associated with phagosomal maturation in macrophages.[27]

To a much lesser extent, punctate cytoplasmic staining was evident for SNAP-23, and may suggest localization to small secretory vesicles. Eosinophil crystalloid granules, however, did not contain detectable levels of either t-SNARE in subcellular fractions. This was confirmed by the apparent absence of SNAP-23 or syntaxin-4

labeling in eosinophil granules by CLSM. A recent report on neutrophils indicated that SNAP-23 is localized to azurophilic and tertiary granules, with minor amounts in plasma membrane-positive fractions.[12] In contrast , we report here that SNAP-23 and syntaxin-4 expression in association with eosinophil granules is negligible. This may indicate that SNAP-23 has distinct roles in membrane trafficking processes in the eosinophil from that of related cell types, e.g., mast cells and neutrophils.

Confocal imaging of t-SNAREs to perinuclear and cytoplasmic regions may correlate with minor peaks of immunoreactivity seen in higher density subcellular fractions (Fig 4.3A). It must be emphasized, however, that the intrinsic buoyant density of the Golgi apparatus, ER, and small secretory vesicles is close to that of plasma membrane using this gradient technique,[26] thus making it difficult to discriminate between these membrane compartments. It is possible that SNAP-23 and syntaxin-4 localized to intracellular sites may participate in membrane trafficking processes. However, the localization of t-SNAREs to these organelles may also simply reflect the presence of newly synthesized proteins destined for the plasma membrane or other cellular locations. Interestingly, a recent report indicated that palmitoylation of conserved SNAP-23 residiues might not be essential for its association with membranes.[31] This may indicate that SNAP-23 is capable of associating with syntaxin family members prior to its own incorporation into membranes.

It is speculated that a critical function for plasma membrane-associated t-SNAREs in eosinophils is in the distal stages of granule/vesicle docking and exocytosis. This prediction is supported by previous observations that SNAP-23 and syntaxin-4 appear to be essential proteins involved in membrane fusion events in

various cell types.[7;12;13;20;28] Based on our findings, using our *in vitro* model of PMD, we propose that granule/vesicle docking and fusion in human eosinophils is absolutely dependent on SNARE complex formation (Fig. 4.5). In this model, small cytoplasmic secretory vesicles are mobilized to the cell membrane. It is conceivable that crystalloid granules transfer portions of their content to cytoplasmic vesicles. Vesicle shuttling, therefore, may provide a mechanism for the selective release of granule-derived mediators during PMD. In addition, we suggest that the t-SNAREs, SNAP-23 and syntaxin-4, participate in docking events during whole granule extrusions. Blocking SNARE molecules may prevent eosinophil degranulation, and thus provide attractive targets for pharmacological manipulation.

Acknowledgements

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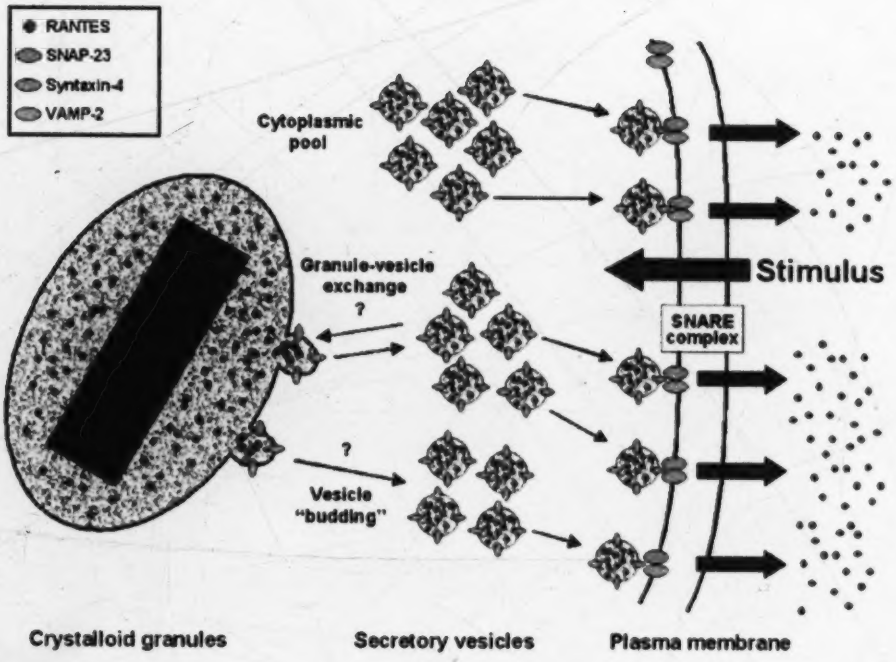

Figure 4.5: Scheme for putative SNARE complex involvement in eosinophil PMD of RANTES.

In resting eosinophils, RANTES is localized to the matrix compartment of crystalloid granules as well as a cytoplasmic pool of VAMP-2$^+$ secretory vesicles. These secretory vesicles serve as a source of rapidly mobilizable RANTES during stimulus-secretion coupling. We hypothesize that VAMP-2$^+$ secretory vesicles may participate in bi-directional trafficking, accepting granule-derived mediators for subsequent transport to the plasma membrane. Alternatively, crystalloid granule membrane "budding" may also contribute to this pool of rapidly mobilizable vesicles. Receptor-coupled stimulation is thought to induce translocation of secretory vesicles to the cell membrane for binding with the t-SNAREs, SNAP-23 and syntaxin-4. The formation of a SNARE fusion complex at vesicle docking is postulated to be an obligatory step for mediator release. (Figure 4.5 was generated by M. Logan)

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Chapter 5

A critical role for tetanus neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP) in human granulocyte exocytosis

Michael R. Logan, Paige Lacy, Solomon O. Odemuyiwa, Melissa Steward, Francis Davoine, Hirohito Kita and Redwan Moqbel

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Abstract

Granulocyte exocytosis is proposed to be critically dependent on the interaction of SNAP receptors (SNAREs) located on granules/vesicles (R-SNAREs) and plasma membrane (Q-SNAREs). Previous studies indicated that the R-SNARE, VAMP-2, as well as Q-SNAREs (SNAP-23, syntaxin-4 and –6) are implicated in exocytosis from human granulocytes. According to the SNARE hypothesis, it is anticipated that distinct R-SNAREs may, in part, regulate mediator release from distinct granule populations. TI-VAMP (VAMP-7) and endobrevin (VAMP–8) have been implicated in endosome/lysosome trafficking, however their function in granulocyte exocytosis remains obscure. We investigated the expression and functional role of SNAREs in the secretion of different granule-derived mediators in human granulocytes. TI-VAMP and VAMP-8, were localized to granule and membrane-enriched fractions, whereas syntaxin-6 was not detectable. In permeabilized cells, anti-TI-VAMP, but not anti-VAMP-8, antibodies impaired the secretion of all mediators examined (in eosinophils, eosinophil peroxidase; in neutrophils, myeloperoxidase, lactoferrin, and matrix metalloprotease-9) in a dose-dependent manner. In contrast, anti-VAMP-2 modestly and selectively impaired secretion from small granules and vesicles. Syntaxin-4, but not syntaxin-6, was found to interact with SNAP-23 and was partially involved in mediator secretion from multiple compartments. Our observations indicate a novel and critical role for TI-VAMP in human granulocyte exocytosis.

Introduction

Exocytosis is dependent on the formation of SNARE (SNAP receptor) complexes during granule docking to the plasma membrane. The SNARE complex is formed by the interaction of one R-SNARE coil, from a vesicle-associated membrane protein (VAMP), with three Q-SNARE coils: one coil from a syntaxin isoform and two coils from either synaptosome-associated-protein of 25 or 23 kDa (SNAP-25 or SNAP-23).[1] VAMP-2 is a critical R-SNARE for synaptic vesicle exocytosis from neurons,[2;3] but is also implicated in secretion in non-neuronal tissues.[4;5] Tetanus (TeNT) and botulinum (BoNT) neurotoxins, which cleave VAMP-2, impair synaptic vesicle exocytosis from neurons.[6] Recent studies indicate that tetanus-insensitive VAMP (TI-VAMP, also known as VAMP-7) and VAMP-8 (endobrevin), are involved in endocytic[7-11] and/or exocytic [12-16] pathways. Both TI-VAMP and VAMP-8 share limited homology with VAMP-2, and are neurotoxin-resistant.[10;12;14]

The release of granule-derived mediators from eosinophils and neutrophils is critical in the manifestation of inflammatory responses in many pathological conditions.[17-20] Pre-formed mediators in eosinophils include cationic proteins stored in crystalloid granules (CG)[21;22] and cytokines, localized to CG and/or secretory vesicles.[23-26] A diverse number of mediators are stored within neutrophils and reside in four distinct granule populations: azurophilic granules (AG), small (secondary) granules (SG), tertiary granules (TG) and secretory vesicles (SV).[27;28]

We have previously shown that VAMP-2 is localized to secretory vesicles in human eosinophils and involved in IFN-γ-induced exocytosis.[29] Brumell *et al* demonstrated that neutrophil VAMP-2 was partially localized to TG and SG, with

negligible expression in AG.[30] Consistent with these findings, Mollinedo *et al* reported VAMP-2 was involved in exocytosis of $CD66b^+$ (formerly CD67) TG and SG but not $CD63^+$ AG in electropermeabilized neutrophils.[31] Both human eosinophils[32] and neutrophils[31;33] express the Q-SNAREs, SNAP-23 and syntaxin-4, which are localized, although not exclusively, to plasma membranes. In electropermeabilized neutrophils, antibody-mediated neutralization of VAMP-2, SNAP-23, syntaxin-4 and -6 leads to loss of SG and TG exocytosis indicating a role for these SNAREs in neutrophil degranulation.[31;33]

The VAMP(s) localized to eosinophil CG and neutrophil AG and their role in exocytosis of these compartments remains to be identified. In addition, it is unclear if specific SNAREs are required for exocytosis of distinct or overlapping granule populations. In this study, we sought to investigate the expression and function of R-SNAREs, VAMP-2, TI-VAMP and VAMP-8, in the secretion of eosinophil and neutrophil granule-derived mediators. We also examined the expression and role of syntaxin-4 and 6 in granulocyte exocytosis, which were previously shown to be important in human neutrophils.[31;33] Our data show a critical role for TI-VAMP in the secretion of stored mediators from multiple granule populations in human granulocytes.

Results

TI-VAMP, VAMP-8 and syntaxin-6 mRNA are expressed in eosinophils and neutrophils.

Eosinophil and neutrophil mRNA was isolated from three subjects and PCR products for TI-VAMP (674 bp), VAMP-8 (587 bp) and syntaxin-6 (549 bp) were generated by RT-PCR (Figure 5.1). Eosinophils from non-atopic donors also expressed mRNA for all three SNARE isoforms (data not shown), indicating gene expression is not exclusive to allergic disease. Cloned PCR products were sequenced which exhibited significant homology (>98%) to reported human cDNA sequences for TI-VAMP, VAMP-8 and syntaxin-6.

TI-VAMP and VAMP-8 are localized to membrane and granule fractions.

Organelles from postnuclear supernatants were separated according to their differential densities by subcellular fractionation, as indicated by peak activity of marker proteins. Eosinophil large CG and small granules were clearly separated from fractions enriched in $CD9^+$ membranes and cytosol (Figure 5.2A). Similarly, neutrophil AG and SG were separated from secretory vesicles/membrane fractions and cytosolic fractions, as indicated by peak activities for MPO, β-HEX, AP, and LDH (Figure 5.2B). Western blot analysis was performed on fractions exhibiting peak activities for granule, membrane and cyosolic markers. TI-VAMP was most abundantly expressed in granule-enriched fractions in both eosinophils and neutrophils, which co-migrated with rat brain positive controls (Figure 5.2C).

VAMP-8 was detected in both granule and membrane fractions which co-migrated with CaCo-2 cell lysate controls (Figure 5.2C) VAMP-8 expression in eosinophils exhibited a similar pattern to that of TI-VAMP, with enrichment in granule fractions. In contrast, we found that VAMP-8 was most strongly expressed in neutrophil secretory vesicles/plasma membrane fractions. In three separate experiments, syntaxin-6 was not detected in either eosinophil or neutrophil fractions despite consistent immunoreactive bands in rat brain controls (Figure 5.2C). The concentration of granule (50-100 μg) or membrane fractions (25-50 μg) for neutrophils were similarly found to contain no detectable syntaxin-6 (data not shown). This observation is in contrast to a previous report demonstrating syntaxin-6 expression in neutrophils.[33]

The expression of TI-VAMP and VAMP-8 in granulocytes was confirmed by flow cytometric analysis of granule fractions which exhibited peak peroxidase activity. Labeling for MBP (69 ± 3%) and CD63 (59% ± 7%) indicated significant enrichment of CG and AG within eosinophil and neutrophil granule fractions, respectively (Figure 5.3A,B). Granule fractions contained negligible plasma membrane contaminants, as indicated by CD9 and alkaline phosphatase (AP) activities for eosinophils and neutrophils, respectively (Figure 5.2). A significant number of eosinophil granules expressed TI-VAMP on their surfaces (22 ± 5%), similarly to AG (20 ± 4%) (Figure 5.3; $p < 0.05$). VAMP-8 was also detected on granules by flow cytometric analysis, with 34 ± 8% expression in CG and 37 ± 6% in AG (Figure 5.3; $p < 0.05$). CG and AG-enriched fractions labeled with VAMP-2 mAb exhibited negligible immunofluorescence above isotype controls (data not shown), as previously

reported.[29-31] No significant immunoreactivity for syntaxin-6 was found on CG and AG granules (5 ± 2% and 6 ± 2%, respectively).

Interaction of SNAP-23 and syntaxin-4 in a *cis*-SNARE complex.

The Q-SNAREs, SNAP-23 and syntaxin-4, localize predominantly to plasma membrane and are involved in exocytosis in several non-neuronal cell types.[34-37] In eosinophils [32] and neutrophils,[31; 33] both isoforms are found at the plasma membrane and other intracellular structures. SNAP-23 and syntaxin-4 interact with multiple R-SNAREs [13; 38;39; 40] and are, therefore, potentially critical docking partners for assembly of *trans*-SNARE complexes (between two membranes) during granule/vesicle docking. In addition, *cis*-SNARE complexes (in the same membrane) have been reported for Q-SNAREs[12;35] which may be important for the regulation of SNARE assembly.

We examined membrane-enriched fractions for SNARE isoforms interacting with syntaxin-4. Membrane fractions of resting eosinophils and neutrophils were subjected to immunoprecipitation with syntaxin-4 mAb. Syntaxin-4 was detected in immunoprecipitates, corresponding with syntaxin-4 in platelet lysate controls. SNAP-23 consistently co-precipitated with syntaxin-4 in granulocytes (Figure 5.4). Similarly, syntaxin-4 co-precipitated with SNAP-23 (data not shown). In membrane fractions generated from granulocytes that had been activated with pCa 5 and 10 μM GTPγS under SLO-permeabilizing conditions for 10 min, SNAP-23 also co-precipitated with syntaxin-4 in activated cells (data not shown). However, analysis of both resting and stimulated immunoprecipitates for VAMP-2, TI-VAMP or VAMP-8 did not reveal immunoreactive bands indicative of a *trans*-SNARE complex due to R-/Q-SNARE

pairing (data not shown). Addition of 1 mM *N*-ethylmaleimide, an inhibitor of SNARE complex disassembly [12;41] either prior to SLO-permeabilization or following stimulation with Ca^{2+} and GTPγS for 10 min, did not enhance the amount of R-SNAREs or SNAP-23 co-precipitated with syntaxin-4. Similar results were obtained for SNAP-23 immunoprecipitates (data not shown).

Effect of SNARE-specific antibodies on secretion of granule-derived mediators.

We determined the role of SNAREs in exocytosis by examining secreted granule proteins from SLO-permeabilized cells incubated in the presence of isoform-specific SNARE antibodies. SLO has been used in studies of Ca^{2+} and GTPγS-mediated exocytosis in guinea pig eosinophils [42;43] and human neutrophils [44;45] and permits the entry of exogenous proteins, including antibodies, into the cell interior.[37;46;47] We confirmed the kinetics of diffusion of exogenous antibodies into permeabilized eosinophils and neutrophils by using flow cytometry analysis. PE-labeled antibodies were shown to rapidly enter (within 1 min) cells incubated with SLO and demonstrated increasing immunoreactivity over time (Figure 5.5A,B). Titration of SLO revealed that neutrophils were more readily permeabilized than eosinophils (0.1 vs 1.0 μg/ml SLO). These doses are comparable to previously reported effective doses, which are known to vary according to cell type, and were used for all subsequent secretion experiments.[37;42;45;47]

In control experiments, GTPγS-induced secretory responses of granulocytes were enhanced by addition of Ca^{2+}, and reached maximal levels within 10 min at pCa 5 (58% ± 14% of total cellular EPO, and 68% ± 8% MPO, $n = 5$). Eosinophils and neutrophils preincubated with isotype antibodies (mIgG1 and rabbit IgG) released

52% ± 9% EPO and 70 ± 9% MPO, respectively, which were similar to controls done in the absence of antibody (n = 6). "Negative controls" (permeabilized at pCa 7) exhibited a small spontaneous release values of EPO and MPO, 9% ± 3% and 14% ± 5%. These observations support a requirement for Ca^{2+} for exocytosis, as previously reported,[43;44] and indicate that SLO did not compromise granule membrane integrity (n = 6) (data not shown). As described in previous studies, granulocytes exhibited a characteristic "rundown" of the secretory response at increasing incubation times with SLO prior to activation.[42;46] Delayed addition of Ca^{2+} and GTPγS to eosinophils markedly reduced the secretion of EPO as early as 5 min, while neutrophil secretion exhibited a more modest impairment which was not evident until ~10 min (Figure 5.5C). To ensure that permeabilized granulocytes exhibited near maximal secretory responses, cells were treated with SLO in the presence of control or SNARE antibodies for 2 min prior to activation with Ca^{2+} (pCa5) and GTPγS for 10 min. As shown in Figure 5.5A & B, this short permeabilization period was sufficient to permit the entry of exogenous antibodies into SLO-permeabilized cells.

We next tested the effects of SNARE antibodies on eosinophil and neutrophil secretion using optimal permeabilization and stimulation conditions. In human eosinophils, VAMP-2 mAb selectively impaired the secretion of EPO to 59 ± 6% of control at an effective dose of 5 μg/ml (Fig. 6A), and inhibition was not potentiated at higher doses (up to 20 μg/ml). In contrast, secretion of EDN (105 ± 11% of control) was unaffected by VAMP-2 mAb (20 μg/ml). In neutrophils, VAMP-2 mAb (up to 20 μg/ml) did not significantly inhibit secretion of MPO and LTF (94 ± 4% and 91 ± 4% of control, respectively). The release of MMP-9 was slightly more susceptible to 5

μg/ml antibody treatment (83 ± 6% of control), although this did not increase using higher doses of VAMP-2 mAb (20 μg/ml) (Figure 5.6B).

We also examined the effect of the enzymatic light chain of botulinum-B (BoNT-B-LC), which targets VAMP-2 but not TI-VAMP.[6;14] Our experiments indicated that 10 nM BoNT-B-LC did not significantly impair the release of either EDN or MMP-9 from eosinophils and neutrophils, respectively (Figure 5.8). We were unable to measure EPO and MPO release since boiled inactivated toxin (at doses of >5 nM) interfered with peroxidase measurements. The presence of dithiothreitol in commercially supplied TeNT and BoNT-B may be responsible for this interference. Similarly, we observed that sodium azide (>0.004%) in commercially supplied antibodies impaired peroxidase assays. Consequently, all antibodies utilized in the study were azide-free.

Anti-syntaxin-4 modestly inhibited secretion of EPO and EDN from permeabilized eosinophils to 74% ± 2% and 76% ± 8% of control, respectively (Figure 5.6A). We observed the same degree of inhibition in neutrophils (~80% of control) for MPO, LTF and MMP-9 in the presence of anti-syntaxin-4 (Figure 5.6B). Similar to responses with VAMP-2 mAb, increasing the dose of syntaxin-4 mAb to 20 μg/ml was not found to enhance its inhibitory effect on any of these mediators. Syntaxin-6 mAb exhibited negligible inhibitory effects on released mediators at 20 μg/ml (Figure 5.6), consistent with our finding that neither eosinophils nor neutrophils expressed detectable syntaxin-6 protein.

Interestingly, TI-VAMP mAb potently impaired the secretion of all granulocyte-derived mediators examined in this study in a dose-dependent manner. In

eosinophils, the secretion of EPO and EDN was significantly inhibited in response to 1 μg/ml TI-VAMP mAb (Figure 5.7A). Similar results were obtained using TI-VAMP mAb for neutrophils, which impaired the secretion of MPO at lower doses (0.2 μg/ml). Slightly higher doses of TI-VAMP mAb were required to inhibit MMP-9 and LTF secretion (0.4 μg/ml) (Figure 5.7B). No inhibitory effect was detectable using VAMP-8 Ab (0.1-20 μg/ml) for any of the tested mediators in granulocytes (Figure 5.7).

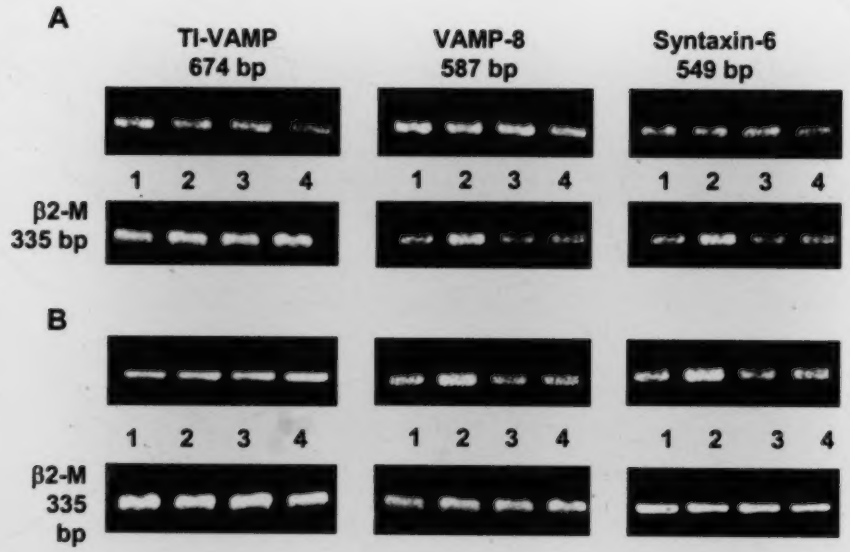

Figure 5.1. Expression of mRNA for TI-VAMP, VAMP-8 and syntaxin-6 in human eosinophil and neutrophils. Total RNA (2 x 10^6 cells) was obtained from three different donors, reverse transcribed and amplified (30 cycles) prior to gel analysis. Products were visualized on ethidium bromide-stained 2% agarose gels. PCR products obtained using SNARE-specific primers for three separate donors (lanes 1-3) for human eosinophils (A) and neutrophils (B). Positive controls for PCR products are shown (Lane 4) using HeLa (syntaxin-6) and CaCo-2 (TI-VAMP, 8) RNA. Each gel shows PCR products for β2-microglobulin control (β2-M) (lanes 1-4, lower panels) (Figure 5.1 was generated by M. Logan)

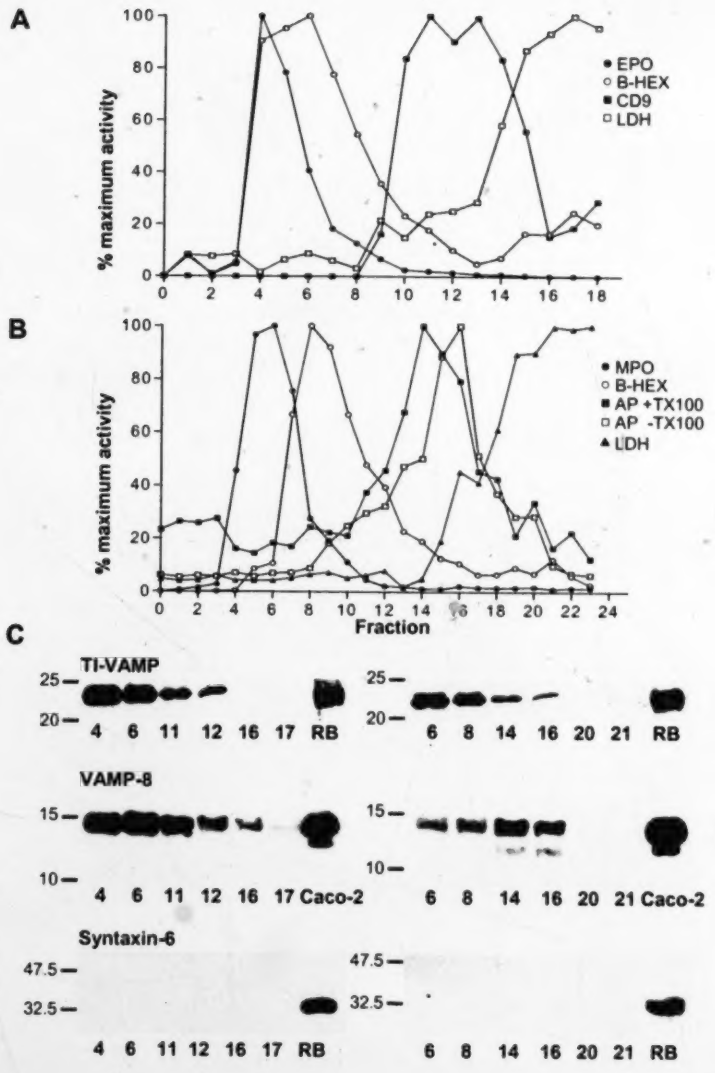

Figure 5.2. Expression of TI-VAMP and VAMP-8 in granule and light membrane fractions. Eosinophil (A)and neutrophil (B) subcellular fractions were generated and examined for marker protein activities: EPO, MPO and β-HEX (granules); alkaline phosphatase (AP for neutrophil membrane/secretory vesicles) and CD9 (eosinophil membrane/secretory vesicles); and LDH (cytosol) as indicated in the Methods section. Fractions with peak activity for marker proteins were examined in duplicate by Western blot analysis for expression of TI-VAMP, VAMP-8 and syntaxin-6: eosinophil fractions (left panels); neutrophil fractions (right panels). Fraction numbers are indicated below each panel and molecular weight markers on the left. Immunoreactive bands are shown in respect to concurrently electrophoresed positive controls: 100 μg rat brain homegenate (RB) (TI-VAMP and syntaxin-6) and 100 μg CaCo-2 cell lysate (VAMP-8).
(Figure 5.2 was generated by M. Logan)

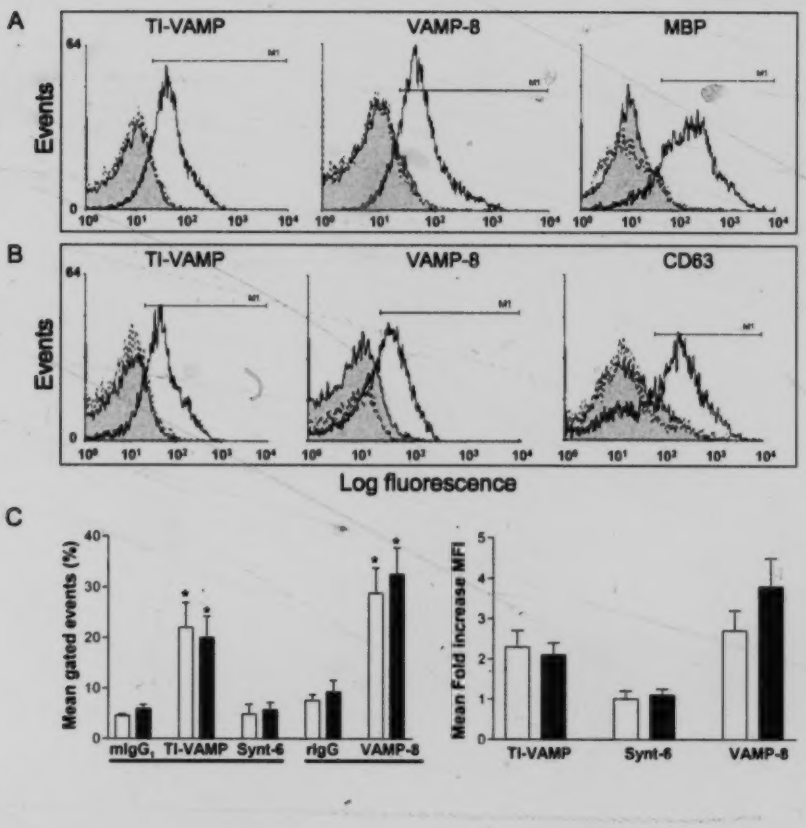

Figure 5.3. Flow cytometric analysis of TI-VAMP and VAMP-8 expression in eosinophil crystalloid (CG) and azurophilic (AG)-enriched fractions. Non-permeabilized eosinophil CG fractions (A) and neutrophil AG fractions (B) were stained with isotype control antibodies (mouse IgG_1 or rabbit IgG; *grey filled*), TI-VAMP or VAMP-8 antibodies (solid *black*). As positive controls, saponin-permeabilized CG were mouse anti-human major basic protein (MBP) mAb and non-permeabilized AG labeled with mouse anti-human CD63 mAb. Negative controls labeled with secondary antibody alone are shown (dotted black). (C) The average ± SEM values for gated events (M1) was calculated for CG (white bars) and AG (black bars) from 3 separate experiments and significance from isotype controls determined (* $p < 0.05$). (D) The mean fold increase of MFI over isotype controls for all CG (white bars) and AG (black bars) populations (10,000 events per measurement) is shown below the flow data. (Figure 5.3 was generated by M. Logan)

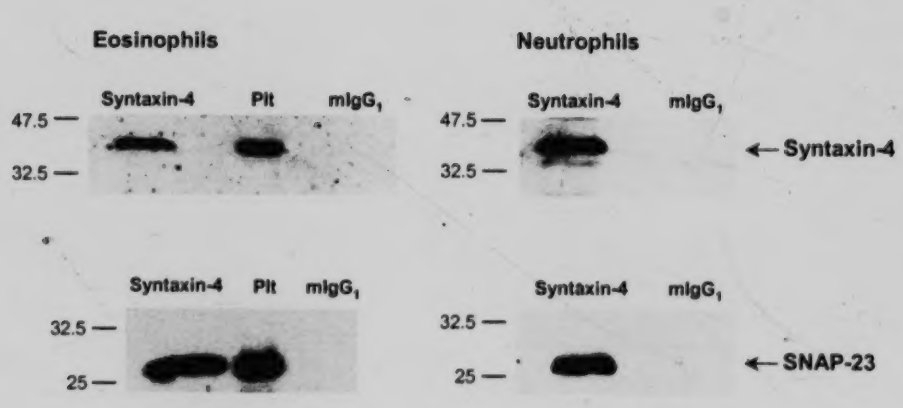

Figure 5.4: In vivo interaction of Q-SNAREs, SNAP-23 and syntaxin-4. Eosinophil and neutrophil light membrane pellets (20 x 10^6 cell equivalents; left and right panels respectively) were precleared using control antibodies ($mIgG_1$) and subjected to immunoprecipitation using syntaxin-4 antibodies. Western blot analyses of precleared light membranes and syntaxin-4 immunoprecipitates are shown using anti-syntaxin-4 (upper panels) or anti-SNAP-23 (lower panels) antibodies. Immunoreactivity of syntaxin-4 and SNAP-23 was confirmed in respect to concurrently electrophoresed positive controls, 50 μg platelet lysate (Plt). Molecular weight markers are shown on the left. (Figure 5.4 was generated by M. Logan)

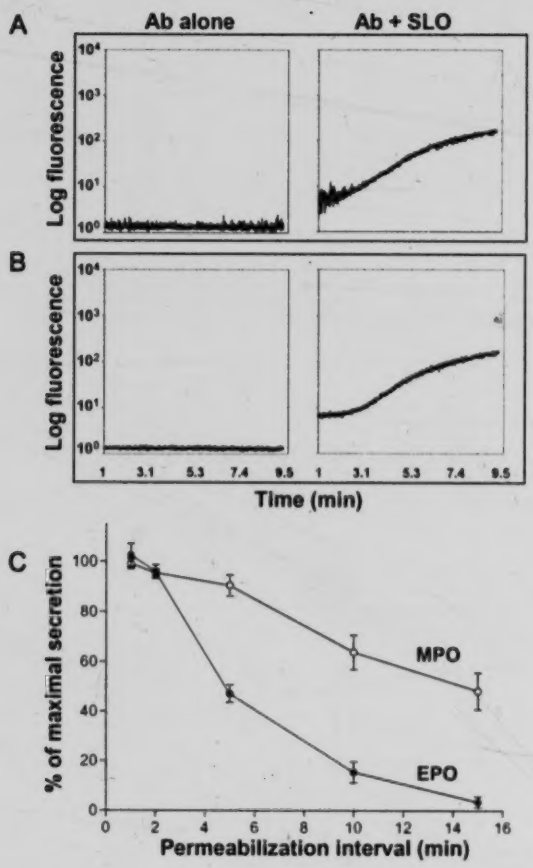

Figure 5.5: Secretory responsiveness following SLO permeabilization and entry of exogenous antibodies into permeabilized granulocytes. Eosinophils (A) and neutrophils (B) were incubated with 1.0 μg/ml PE secondary antibodies in the absence or presence of 1.0 μg/ml or 0.1 ug/ml SLO, respectively, for 1 min at 37°C. Cells were then examined continuously by flow cytometery for the change in log fluorescence intensity for ~8.5 min. (C) Exocytosis of EPO and MPO was determined for eosinophils and neutrophils, respectively, after increasing periods of permeabilization with SLO (permeabilization interval) prior to activation with Ca^{2+} (pCa 5) and 10 μM GTPγS. Values are expressed as percentage of maximal release which was detected at the time = 0 permeabilization interval (58% ± 14% EPO and 68% ± 8% MPO of total cellular peroxidase activity). (Figure 5.5 was generated by M. Logan)

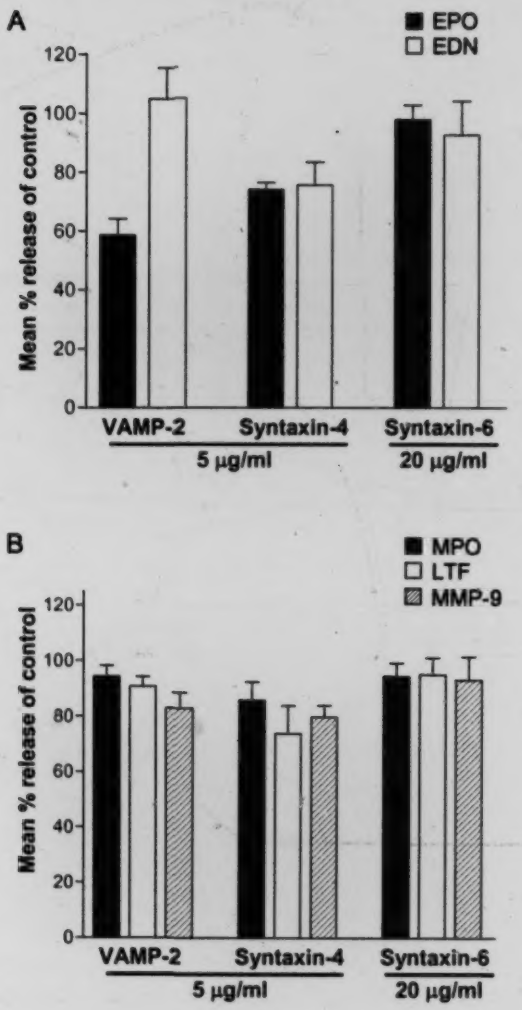

Figure 5.6: Effect of VAMP-2- and syntaxin-4-specific antibodies on granule-derived mediator secretion. Human eosinophils (A) and neutrophils (B) were permeabilized in SLO and incubated in the presence of isotype control antibodies ($mIgG_1$), VAMP-2 mAb, syntaxin-4 mAb or syntaxin-6 mAb (0.1-20 µg/ml) prior to activation with 10 µM Ca^{2+} and GTPγS for 10 min. Cell supernatants were examined for granule-derived mediators as indicated in Methods. Data are expressed as the average percentage of isotype control ± SEM from three separate experiments. Shown are representative averaged experiments for an effective dose of 5 µg/ml which represents the maximum inhibitory response for these antibodies.
(Figure 5.6A was generated by M. Logan. Figure 5.6B was generated by M. Steward)

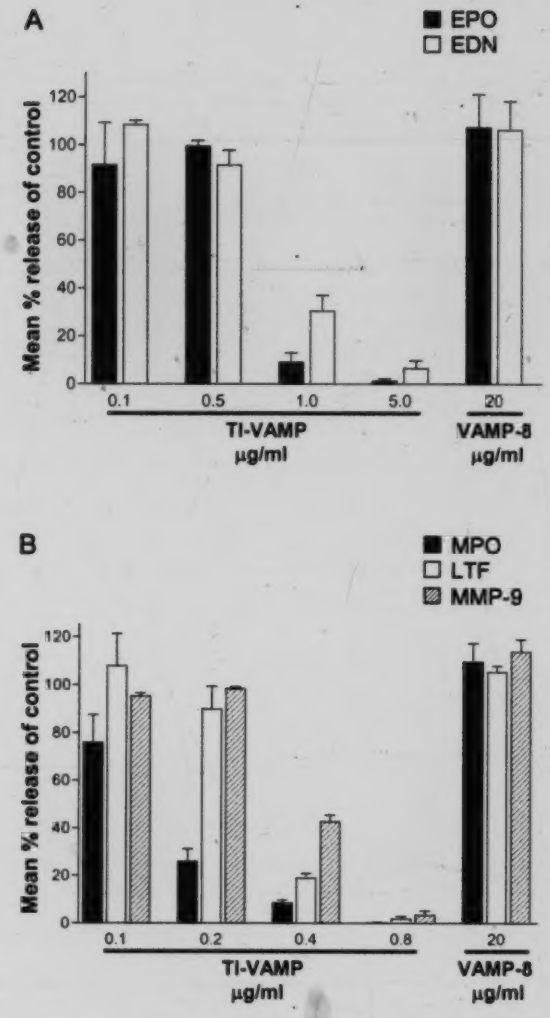

Figure 5.7: Effect of TI-VAMP-specific antibodies on granule-derived mediator secretion. SLO-permeabilized eosinophils (A) and neutrophils (B) were incubated in the presence of increasing doses of TI-VAMP mAb, VAMP-8 Ab or matched concentrations of isotype control antibody ($mIgG_1$ or rabbit IgG), activated with Ca^{2+} and GTPγS for 10 min, and secretion of granule-derived mediators was determined as described in the legend for Figure 5.6. (Figure 5.7 was generated by M. Logan)

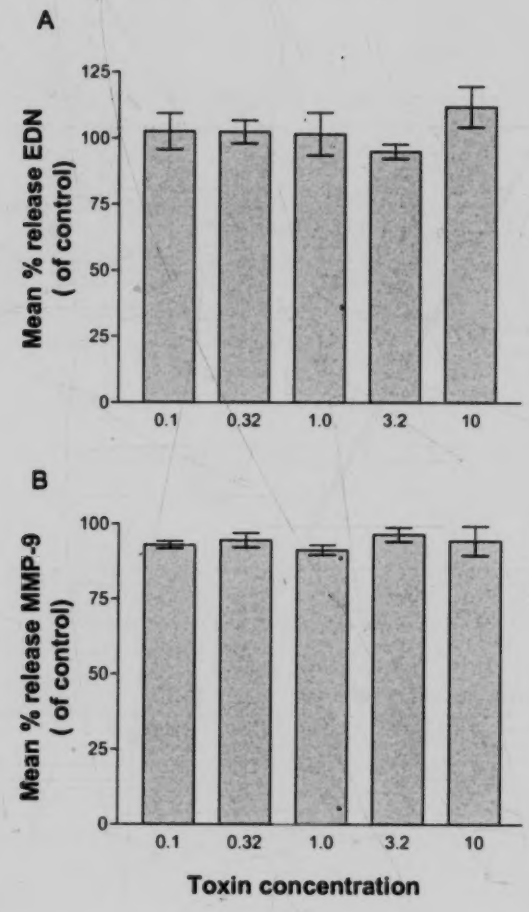

Figure 5.8: Effect of Botulinum B enzymatic light chain (BoNTB-LC) on granule-derived mediator secretion. SLO-permeabilized eosinophils (A) and neutrophils (B) were incubated in the presence of increasing doses of BoNTB-LC (nM) or matched concentrations of heat inactivated toxin (boiled for 5 min), activated with Ca^{2+} and GTPγS for 10 min, and secretion of (A) eosinophil derived neurotoxin (EDN) and (B) matrix metalloproteinase-9 (MMP-9) determined. (Figure 5.8A was generated by M. Logan. Figure 5.8B was generated by M. Steward)

Discussion

Our results demonstrate for the first time that TI-VAMP is critically involved in exocytosis of granule-derived mediators from human granulocytes. We show that eosinophil CG and neutrophil AG express significant amounts of TI-VAMP and VAMP-8 on their granule membranes. Examination of subcellular fractions indicated that TI-VAMP is not restricted to CG and AG, however, and is also expressed in secretory vesicle/plasma membrane-enriched fractions. A functional role for TI-VAMP in exocytosis of CG and AG is implicated in the finding that EDN and MPO secretion were abolished in the presence of TI-VAMP mAb. The observation that TI-VAMP mAb inhibited the release of neutrophil SG and TG mediators, in particular, suggests that TI-VAMP is not restricted to a single compartment and may participate in exocytosis of more than one granule population.

Exocytosis in neutrophils has been reported to exhibit a hierarchical pattern, with rapid mobilization of small vesicles, SG and TG followed by the slower release of AG.[48] Eosinophils exhibit rapid mobilization of secretory vesicles, termed piecemeal degranulation (PMD),[24;49-51] which may or may not coincide with CG exocytosis depending on the agonists utilized.[24;25;52-54] Our observations confirm previous studies[52;53;55;56;57] that Ca^{2+} and GTPγS-induced activation of eosinophils and neutrophils induces exocytosis of multiple granule compartments. In accordance with the SNARE hypothesis, it is postulated that exocytosis of distinct granule populations may be regulated, in part, by different SNARE isoforms.[58]

Antibody and/or peptide neutralization of SNAREs is proposed to result in conformational and/or steric hindrance of SNARE assembly, and has been

demonstrated to impair secretory responses from a variety of cell types.[31;34;36;59;60] The TI-VAMP mAb used in this study binds specifically to the cytoplasmic region of TI-VAMP which includes the domain required for SNARE assembly.[40] In contrast to our findings with TI-VAMP mAb, the application of VAMP-2 mAb only partially inhibited EPO but not EDN secretion. EPO and EDN are localized to CG in eosinophils,[61;62] although immmunoreactivity for EPO has also been observed in small cytoplasmic vesicles by electron microscopy.[51] We have previously shown that VAMP-2 immunoreactivity coincided with RANTES-containing small secretory vesicles, but is not detectable on large crystalloid granules in human eosinophils.[29] The selective inhibitory effect of VAMP-2 mAb on secreted EPO in the present study suggests that, under these experimental conditions, a proportion of exocytosed EPO is mediated by small vesicles.

Hoffmann *et al.* similarly reported that VAMP-2 was enriched in fractions containing secretory vesicles but not CG. They observed that TeNT impaired the release of ECP in SLO permeabilized eosinophils, although this was not compared to inactivated toxin.[64] In contrast to their findings, we observed that the release of the CG mediator, EDN, was not blocked by 10 nM BoNT-B light chain which cleaves VAMP-2 at the identical site as TeNT. As well, we observed that anti-VAMP-2 treatment only partially blocked EPO release which was not potentiated at higher doses of antibody. The differences in experimental approaches to both SLO permeabilization, cell activation and examined mediators may account for this discrepancy. In addition, our present study has shown that caution should be used in

the use and interpretation of toxin-mediated effects, since increasing doses have the potential to interfere with the measurement of secreted mediators.

Similar to eosinophils, VAMP-2 was localized to neutrophil secretory vesicles and/or small granules in previous studies.[30;31] Mollinedo *et al* demonstrated that antibody-mediated neutralization of VAMP-2 impaired the surface upregulation of the SG/TG marker, CD66b, but not the AG marker, CD63, in electropermeabilized neutrophils.[31] Consistent with this study, we observed that the same VAMP-2 mAb had a negligible inhibitory effect on the release of AG-derived MPO. In contrast to this study, which indicated VAMP-2 mAb substantially blocked CD66b upregulation, we observed a very modest inhibitory effect on secreted LTF and MMP-9 using the same antibody. It is possible that this discrepancy may relate to differences in experimental approaches of permeabilization and the evaluation of exocytosis. In addition, CD66b was recently identified is a common marker for SG and TG.[31] Since we have determined the differential exocytosis of mediators stored in SG and TG, this may also account for our reported differences using VAMP-2 mAb.

In contrast to our VAMP-2 mAb results, antibodies targeting TI-VAMP abolished the release of neutrophil MPO, LTF and MMP-9 in a dose-dependent manner. Higher doses of TI-VAMP mAb were required to impair neutrophil LTF and MMP-9 release compared to MPO release. This may reflect differences in epitope availability during cell activation and/or the temporal progression of the secretory response. The sequential release of SG/TG followed by AG has been associate with a biphasic response in Ca^{2+} sensitivity.[44;65] Thus, it is possible that antibody-mediated inhibition of AG is more effective at lower doses due to their slower recruitment to the

plasma membrane. The observation that TI-VAMP mAb potently blocked LTF and MMP-9 release indicates that this R-SNARE is involved in exocytosis of SG and TG, in addition to AG.

The expression of TI-VAMP on CG and AG reported here is consistent with observations that this R-SNARE is localized to $CD63^+$ compartments in rat basophilic cells (RBL-2H3) and late endosomes/lysosomes in other cell types.[7;40;59;66] Rao *et al* recently demonstrated that exocytosis of lysosomes in fibroblasts is mediated by TI-VAMP.[59] Recent studies have revealed insights into the mechanism of sorting TI-VAMP to $CD63^+$ compartments. Deletion of the amino terminal domain of TI-VAMP (longin domain) was shown to cause its mis-sorting from late endosomes to the apical membrane in transfected fibroblasts.[40] The longin domain was demonstrated to interact with a subunit of the AP-3,[40] an adaptor protein which has been demonstrated to be critical for the sorting of membrane proteins such as CD63 (LAMP-3), LAMP-1 and LAMP-2 to lysosomes.[67;68] TI-VAMP does not appear to be restricted to lysosome trafficking, however, and was reported in vesicle trafficking to the apical membrane in neurons[15;16] and epithelial cells.[14]

The localization of VAMP-8 to CG and AG suggests a potential role in exocytosis. VAMP-8 was previously implicated in platelet granule exocytosis [13] and is localized to granules in RBL-2H3 cells.[12] However, VAMP-8 has been localized to the *trans*-Golgi network and early endosomes [8-11] in other cell types, suggesting its function may be cell type-specific. In our hands, VAMP-8 Ab did not impair the release of any of the tested granule proteins. This may indicate VAMP-8 is not functionally implicated in eosinophil and neutrophil exocytosis. However, it is

possible that the accessibility to the VAMP-8 epitope may be limited *in situ* and further studies are needed to determine if this R-SNARE participates in granulocyte exocytosis.

The Q-SNAREs, SNAP-23 and syntaxin-4, are expressed in eosinophils [32] and neutrophils[30;31;33] and are potential docking partners for R-SNAREs during exocytosis. Antibody-mediated inhibition of syntaxin-4 modestly impaired the secretion of all neutrophil and eosinophil mediators measured in this study. Similar to the effects of the VAMP-2 mAb, we found that increasing doses of syntaxin-4 mAb did not potentiate the inhibitory effects on secretion from either cell type. It is plausible that SNAP-23/syntaxin-4 *cis*-SNARE complexes, which were detected in bothgranulocytes, may limit epitope availability of syntaxin-4 during activation, thereby reducing the inhibitory effect of the syntaxin-4 mAb.

We attempted to immunoprecipitate *trans*-SNARE complexes, but were unable to detect R-SNAREs VAMP-2, TI-VAMP, or VAMP-8 in syntaxin-4 immunoprecipitates in either resting or Ca^{2+}/GTPγS-activated cells (with or without *N*-ethylmaleimide), although SNAP-23 readily co-precipitated with syntaxin-4. In neurons, *trans*-SNARE complexes are detectable during active exocytosis, although these are rapidly disassembled and VAMPs are recycled within seconds from the plasma membrane.[69] It is possible that the rapid process of SNARE recycling events may have limited the numbers of stable R/Q-SNARE pairings, thereby preventing immunoprecipitation of *trans*-SNARE complexes in granulocytes. It is also possible that the epitopes recognized by the syntaxin-4 and SNAP-23 antibodies are concealed

within *trans*-SNARE complexes and/or by additional regulatory molecules associated with Q-SNAREs during activation of exocytosis.

Syntaxin-6 has been shown to partner with several SNAREs and is localized to several sites including the *trans*-Golgi network,[70] secretory granules/vesicles,[71] endosomes [72] and plasma membrane.[33] In our studies, syntaxin-6 mRNA was expressed in both human eosinophils and neutrophils from separate donors. Using commercially available antibodies, we found negligible syntaxin-6 protein in cell lysates and membrane fractions from either cell type. Consistent with this observation, we observed that syntaxin-6 mAb did not impair secretion of any of the examined mediators in permeabilized cells. Our findings are in contrast to an earlier study which indicated a role for syntaxin-6 at neutrophil plasma membranes in the exocytosis of $CD66b^+$ and $CD63^+$ granules.[33] The discrepancy between our findings and this previous study may reflect differences in experimental approaches and/or the syntaxin-6 antibodies used. However, an alternative explanation is that very low/neglible levels of syntaxin-6 observed may relate to the regression of the Golgi apparatus in mature granulocytes.[73;74]

In summary, our observations support a role for TI-VAMP in exocytosis of $CD63^+$ granules, which have been previously proposed as "secretory lysosomes," [75] and small granule/vesicle populations in eosinophils and neutrophils. The observation that TI-VAMP is involved in mediator release from different granule compartments is consistent with recent observations that SNAREs are capable of interacting with multiple partners[13;39;76], and have the potential to participate in more than one trafficking step.[77;78] Further studies are required to determine the signaling molecules

involved in the sequential recruitment of granule populations, a process which may involve various SNARE isoforms. These studies are necessary in the pursuit of candidate proteins that may prove to be selective targets for the modulation of secretory function from these important inflammatory cells.

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CHAPTER 6

I. Summary and model of SNARE-mediated exocytosis in eosinophils and PMNs

The observations arising from this research project, in agreement with the SNARE hypothesis [1], have provided evidence for a critical role for SNARE isoforms in exocytosis of stored mediators from human eosinophils and PMNs. As described previously, the intracellular application of Ca^{2+} and GTPγS into SLO-permeabilized granulocytes is an effective albeit artificial method to induce secretion from these cell types.[2-5] A modification of this SLO-permeabilization technique, used for the described studies (Chapter 5), is an effective method to introduce neutralizing antibodies into the cell interior that interfere with SNARE function. Although this technique has its limitations, particularly due to the loss of cell responsiveness following permeabilization, it is a useful tool to determine the role of candidate proteins involved in exocytosis using antibodies and/or peptide inhibitors. The data I have presented support a major role for TI-VAMP in exocytosis of large and small granules from eosinophils and PMNs. In contrast, VAMP-2 is restricted to small vesicles and does not appear to play a major role in exocytosis of large CG and AG from eosinophils and PMNs, respectively. The Q-SNAREs, SNAP-23 and syntaxin-4 have been identified as docking partners of VAMP-2 and TI-VAMP for exocytosis in these granulocytes (Figure 6.1).

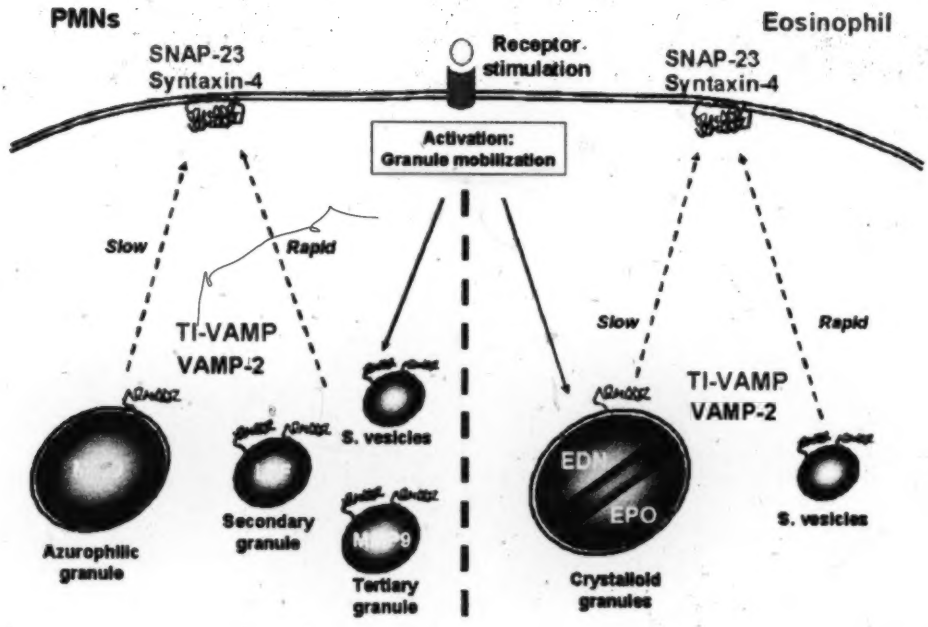

Figure 6.1: Model SNARE isoforms implicated in eosinophil and PMN exocytosis. The R-SNARE, VAMP-2, was localized to small secretory vesicles in both eosinophils and PMNs (Chapter 5). In addition, VAMP-2 was reported to be expressed on PMN secondary and tertiary granules (Mollinedo et al. 2003. J. Immunol 170 (2) 1034-42). Our data suggested VAMP-2 is mostly involved in the rapid release of small vesicles following receptor-induced cell activation (Chapters 3, 5). In particular, VAMP-2 was shown to be a candidate R-SNARE involved in eosinophil piecemeal degranulation (PMD). TI-VAMP was localized to PMN azurophilic granules and eosinophil crystalloid granules, and is involved in the release of stored mediators from these compartements. TI-VAMP was not restricted to large granules, and antibody-mediated inhibition of TI-VAMP supported that it is involved in the release of PMN secondary and tertiary granule mediators (Chapter 5). The Q-SNAREs, SNAP-23 and syntaxin-4, were demonstrated to be docking partners for VAMP-2 and TI-VAMP at the plasma membrane (Chapter 4). The R-SNARE, VAMP-8 (not shown), was localized to PMN azurophilic granules and eosinophil crystalloid granules. In addition, it was also expressed in membrane-enriched fractions. Antibodies directed to VAMP-8 did not impair the release of any granule mediators examined, which may suggest VAMP-8 is not involved in exocytosis of storage granules (Chapter 5). Further studies are needed to determine if VAMP-8 is involved in granule-granule fusions or granulogenesis in these cells.

R-SNAREs

This study provided the first demonstration that TI-VAMP is localized to $CD63^+$ eosinophil CG and neutrophil AG, and involved in exocytosis of these compartments. TI-VAMP was also expressed in membrane-enriched compartments in both cell types, indicating it is not exclusively localized to large granules. The observation that antibodies directed to TI-VAMP impaired the secretion of distinct markers of AG, SG and TG in permeabilized PMNs supports this R-SNARE is involved in the trafficking of more than one granule compartment. The multiple role of TI-VAMP is plausible particularly in light of recent studies in yeast that have demonstrated a single SNARE isoform may be involved in multiple trafficking steps *in vivo*.[6-8] Although VAMP-8 was localized to both granules and enriched membrane fractions, similar to TI-VAMP, antibodies directed to VAMP-8 did not exhibit any inhibitory effect on granule mediator release. As mentioned in Chapter 5, we cannot rule out that VAMP-8 antibodies were unable to bind to their eptiopes *in vivo* and, therefore, were not effective to block VAMP-8.

Recent studies by Galli and colleagues have provided compelling evidence that TeNT-resistant exocytotic events required for the elongation of axons and dendrites are critically dependent on TI-VAMP during nerve development.[9;10] As might be anticipated, TI-VAMP immunoreactivity in developing neurons does not coincide with $VAMP\text{-}2^+$ compartments, supporting the notion that distinct sets of R-SNAREs mediate vesicle/granule trafficking.[11] TI-VAMP is partially colocalized to $CD63^+$ compartments in PC12 neurons [11] and is similarly localized to $CD63^+$ late endosomes (LE) and lysosomes (LYS) in other non-neuronal cells.[12-15] Andrews and colleagues

recently demonstrated that TI-VAMP is localized to LYS in NRK cells. TI-VAMP-dependent exocytosis of LYS is triggered in response to plasma membrane injury and the elevation of intracellular calcium.[13] This process provides a mechanism to maintain cell viability by inserting a membrane "patch" into the plasma membrane.[16;17] Membrane injury induced by eosinophil cationic proteins (MBP, ECP, EDN) may provide a similar mechanism to stimulate degranulation in a paracrine or autocrine fashion.[18;19] For example, it has recently been shown that MBP induced both O_2^- production and IL-8 release from PMNs via a phosphoinositide 3-kinase (PI3K) and PKC-zeta-dependent signaling mechanism.[20]

A number of studies have shown that VAMP-2 is the dominant R-SNARE involved in synaptic vesicle release from neurons [21-23] and in exocytosis from non-neuronal cells.[24-27] We showed VAMP-2 was expressed in membrane-enriched fractions with no detectable immunoreactivity in CG-enriched fractions from human eosinophils.[28] In resting cells, VAMP-2 immunoreactivity coincided with RANTES (previously demonstrated to be localized to SV and the matrix of CG [29]) and was distributed throughout the cytoplasm. Following stimulation with IFN-γ, which was shown previously to mobilize RANTES-containing SV, immunoreactivity for both VAMP-2 and RANTES was shifted rapidly to the cell periphery.[28] Although we were unable to examine the secretion of RANTES in SLO-permeabilized eosinophils (discussed in Chapter 2), anti-VAMP-2 antibodies partially inhibited Ca^{2+} and GTPγS-induced secretion of EPO but had no effect on EDN release. EPO has been previously localized to SV [30;31], in addition to its localization to CG. Collectively, these

observations support a role for VAMP-2 in SV exocytosis during PMD, but do not indicate a role for this R-SNARE in CG fusions by classical exocytosis.

Similar to eosinophils, we found negligible protein expression for VAMP-2 in AG-enriched fractions. VAMP-2 immunoreactivity was detected in membrane-enriched fractions, albeit not at high levels. Antibody-targeted inhibition of VAMP-2 did not appreciably block the release of marker proteins from AG, SG or TG in SLO-permeabilized PMNs. These observations suggest that VAMP-2 is predominantly involved in small vesicle trafficking in these cells. In support of this notion, Brumell *et al* (1995) previously showed that VAMP-2 was found mostly in secretory vesicle/plasma membrane enriched fractions and, to a lesser exent, TG fractions from human PMNs. VAMP-2 immunoreactivity did not coincide with CD63 and CD67 (now designated CD66b), but co-localized to CD35, a marker of SV.[32] In another study by Mollinedo *et al*, VAMP-2 was localized to some, but not all, SG and TG by immunogold electron microscopy. In this study, the same VAMP-2 mAb used in our experiments impaired exocytosis of $CD66b^+$ SG and TG, but not $CD63^+$ AG in electropermeabilized PMNs. TeNT (400 nM) was found similarly to impair CD66b surface upregulation, although an inactivated toxin control was not utilized nor CD63 examined.[33] These observations suggested VAMP-2 is involved in SG and TG exocytosis, although it is unlikely to be a specific marker for these granules. We observed that BoNTB-LC (10 nM), which cleaves VAMP-2 at the same site as TeNT, did not impair the release of either EDN or MMP-9 from eosinophils and PMNs, respectively. While it is possible that higher doses of either BoNTB-LC or TeNT may impair secretory function in eosinophils and PMNs, our studies have shown that the

conclusions from these experiments should be approached carefully. We observed that even low concentrations of boiled inactivated toxin (>20 nM) interfered with the peroxidase (EPO/MPO) assay and, therefore, can interfere with the readout of secreted mediators. It is possible that the high level of dithiothreitol (DTT) (10 mM) in commercially supplied BoNT and TeNT (required to reduce and activate the toxin) may be responsible for this effect.

Q-SNAREs

We have observed that both SNAP-23 and syntaxin-4 are exclusively localized to membrane-enriched fractions from eosinophils and not detected in granules. Both Q-SNAREs were localized predominantly to the plasma membrane, with additional immunoreactivity for SNAP-23 and syntaxin-4 in the Golgi compartment and ER, respectively.[34] Anti-syntaxin-4 antibodies modestly suppressed exocytosis of mediators from eosinophils and PMNs, which may have been attributed to limited epiotpe availability of syntaxin-4 due to its interaction with SNAP-23 (Chapter 5). In a previous study, Martin-Martin *et al* reported that SNAP-23 antibodies impaired exocytosis of $CD67^+$ (now CD66b) small granules (SG/TG), but not $CD63^+$ AG, in electropermeabilized PMNs.[35] However, it is unclear how this selective effect occured, particularly since SNAP-23 was detected in membrane-enriched fractions, SG, TG and, to a much lesser extent, AG of resting PMNs.[35] In this same study, syntaxin-6 was detected in membrane fractions and interacted with SNAP-23. Antibody-mediated inhibition of syntaxin-6 in electropermeabilized PMNs was reported to block both the surface upregulation of CD63 and CD66b, indicating a role

in both small (SG and TG) and large (AG) exocytosis.[35] Our studies, utilizing commercially available azide-free monospecific antibodies, did not reveal any detectable syntaxin-6 protein in either eosinophils or PMNs. These findings were in agreement with the observation that syntaxin-6 antibodies (up to 20 μg/ml) did not impair the release of any of the examined eosinophil or PMN mediators examined in SLO-permeabilized cells (Chapter 5).

II. Potential mechanisms of TI-VAMP and VAMP-8 function in granule/vesicle trafficking and granule biogenesis

TI-VAMP and VAMP-8 in the endocytic pathway

A number of recent studies have supported the notion that TI-VAMP and VAMP-8 are involved in endocytic transport to the LE and LYS. TI-VAMP is widely expressed in several rat tissues [14] and, as mentioned in section I, is localized to $CD63^+$ LE and LYS or lysosome-like storage granules in various cell types.[12-15] In addition, TI-VAMP has been shown to localize to transport vesicles in epithelial cells [36] and developing neurons [9-11] as well as the *trans*-Golgi network (TGN) by electron microscopy in PC12 neurons.[14] VAMP-8 has been shown to preferentially localize to EE [37-39] and has since been observed on compartments that communicate with these organelles. These include the plasma membrane, clathrin-coated pits, LE and the TGN.[40;41]

Studies using neutralizing antibodies and/or recombinant proteins have supported a role for TI-VAMP in transport to the LE and LYS. The addition of the

cytoplasmic domain of TI-VAMP was found to impair homotypic fusions of LYS and LE-LYS derived from rabbit alveolar macrophages, *in vitro*. This treatment had no effect on the homotypic fusion events of early endosomes (EE), suggesting TI-VAMP is selectively involved in LE and LYS fusion events.[15] Similarly, anti-TI-VAMP antibodies introduced into SLO-permeabilized HeLa cells impaired the degradation of internalized epidermal growth factor (EGF) without affecting the internalization of EGF or recycling of the transferrin receptor to the plasma membrane.[14]

Anti-VAMP-8 antibodies were reported by Antonin *et al* (2000) to impair the homotypic fusion of EE and EE-LE fusions, *in vitro*. [40] Interestingly, it was mentioned in a separate study by Mullock *et al* (2000) that VAMP-8 antibodies exhibited no inhibitory effect on LE-LYS fusions *in vitro*.[41] Our findings that VAMP-8 antibodies did not impair mediator release from lysosome-like eosinophil CG and neutrophil AG similarly suggest that VAMP-8 may not be involved in fusion events from LYS and/or storage granules. VAMP-8 was also detectable in membrane fractions, suggesting it may be localized to SV and/or endosomes. It is possible that VAMP-8 is acquired by CG and AG during their development, possibly through interaction with endosomes (Figure 6.2). Further studies are required to determine the role of VAMP-8 in these cells.

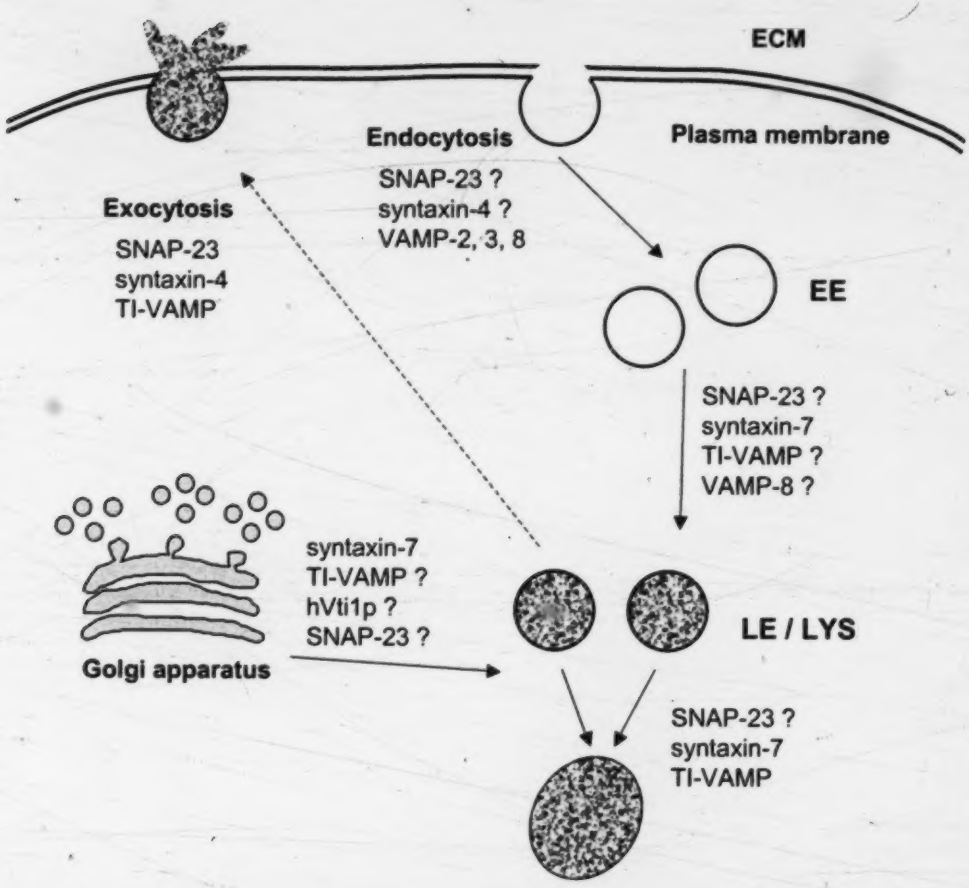

Figure 6.2: Model of SNARE isoforms involved in the endocytic pathway. R-SNARES localized to endosomes in mammalian cells include VAMP-2, -3 and –8. The plasma membrane Q-SNAREs, SNAP-23 and syntaxin-4 may be involved in endocytosis. Transport from early endosomes (EE) to late endosomes (LE) and/or lysosomes (LYS) has been shown to be mediated by TI-VAMP and/or VAMP-8. The majority of experimental data supports that TI-VAMP is involved in homotypic fusions of LE/LYS. The Q-SNARE, syntaxin-7 has been demonstrated to be involved in transport from EE to LE/LYS and homotypic fusions of LE/LYS. Syntaxin-7 has also been shown to be involved in the delivery of lumenal proteins to LYS. TI-VAMP and SNAP-23 have been localized to the trans-Golgi network, in addition to other intracellular sites in mammalian cells. This suggests that these isoforms may be involved in delivery of lumenal proteins to LYS, similar to syntaxin-7. In addition, a human homologue of the yeast R-SNARE, Vti1p, hVti1p has been identified recently. Vti1p is essential for the development and maturation of yeast lysosome-like vacuoles. It is likely that hVti1p is similarly required for LYS development in mammalian cells. Exocytosis of TI-VAMP containing compartments, such as LYS or LY-like storage granules has been shown to be mediated by TI-VAMP, SNAP-23 and syntaxin-4 (Chapter 5, Rao. *et al.* 2004. J. Biol. Chem. 279(19) 20471-79)

According to the SNARE hypothesis, in order for homotypic LYS or LE-LYS fusion to proceed, Q-SNAREs must be present on these compartments. Our observations (Chapter 5), and those of Rao *et al* (2004) [13], have indicated that exocytosis of TI-VAMP-containing compartments is mediated by the Q-SNAREs, SNAP-23 and syntaxin-4. As discussed in section II, SNAP-23 is localized to several intracellular sites including membrane channels, granules and plasma membranes in different hematopoeitic cell types. SNAP-23 has also been reported on endosomal membranes and the TGN, suggesting it could participate in LE/LYS trafficking and the biosynthetic pathway (Figure 6.2).[42;43] In eosinophils, SNAP-23 and syntaxin-4 were undetectable in CG-enriched fraction, which suggests that other Q-SNARE isoforms may be involved in granule-granule fusions during maturation and compound exocytosis.[34]

Syntaxin-7 has recently been shown to localize to LE and LYS and is capable of associating with both TI-VAMP [44] and VAMP-8.[41;44] Antibodies directed against syntaxin-7 [15;41] and the syntaxin-7 cytoplasmic domain [15] have been shown to impair LE-LYS fusions, *in vitro*. In addition, the overexpression of the cytoplasmic domain of syntaxin-7 impaired the degradation of internalized FITC-dextran in NIH3T3 and NRK cells. Overexpression of this mutant did not influence the internalization process or the transport of cathepsin D and LAMP-2 to LYS. Thus, it is postulated that syntaxin-7 is predominantly involved in transport from EE to LE without influencing the biosynthetic transport pathway from the TGN.[45] Syntaxin-7 has been shown to be expressed in human platelets. However, antibody-mediated inhibition of syntaxin-7 was found to not affect mediator release significantly from either platelet dense

granules [46] or lysosomes.[47] The role of syntaxin-7 in granule biogenesis and exocytosis from eosinophils and PMNs remains to be determined.

A conserved mechanism for lysosome/granule biogenesis and secretion?

TI-VAMP is a member of a highly conserved family of proteins termed "*longins*". *Longins* contain ~110 additional amino acids in the N terminus that are not found in other VAMP species (*brevins*). An 80% sequence homology of the longin domain is observed for 77 of the 110 residues across yeast, protozoans, animals and plants (Figure 6.3). Interestingly, sequencing of the genomes of *Arabidopsis thaliana*, as well as a number of other plant species have revealed no *brevin* but several *longin* genes.[48] The observations of Galli and colleagues that exocytosis mediated by TI-VAMP is critical for neuron development support an essential function for this isoform in animals.[9;10] In addition, studies on the development and fusion events of yeast vacuoles suggest that biogenesis and fusion of LYS and, potentially, LYS-like granules such as eosinophil CG and neutrophil AG may be mediated by a conserved protein machinery.

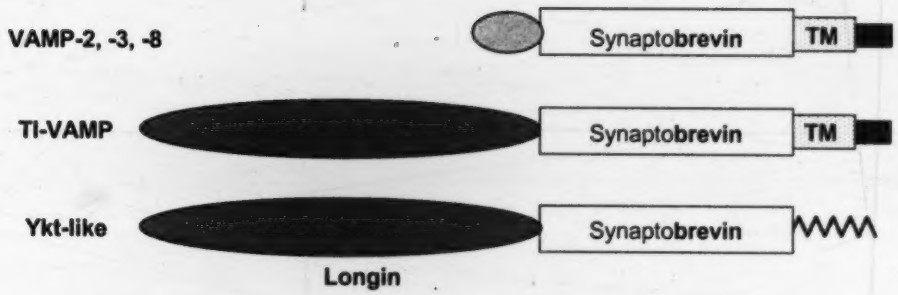

Figure 6.3: Structure and domain composition of brevins and longins. In addition to the the conserved synaptobrevin (or **brevin**) domain common to all VAMP species, tetanus-insensitive VAMP (TI-VAMP) and related isoforms (eg: Ykt-lik efamily members such as the *S. cerevisiae* R-SNARE, Ykt6p) contain an additional amino terminal region of approximately 110 residues termed the **longin** domain (striped oval). Longins are found in protists, plants and animals suggesting an important and evolutionary conserved function. Brevins (such as VAMP-2, -3 and –8) and the longin, TI-VAMP both have transmembrane (TM) spanning anchors. YKT-like longins have an isoprenyl anchor (zig-zag line) instead of a transmembrane domain (TM). The grey oval in the structure of brevins represents a variable N-terminus and the black rectangles at the C-termini of brevins and longins represent intravesicular tails. Modified from: *Filippini F. et al. 2001. Trends. Biochem. Sci. 26(7) 407-409.*

Analysis of the sequence of the yeast vacuole R-SNARE, Ykt6p, has pointed to it as a member of the *longin* family, similar to human TI-VAMP.[48] Syntaxin-7 was first identified for its homology with the yeast TGN Q-SNARE, Pep12p [49] and was later shown to exhibit a higher homology with the Q-SNARE, Vam3p in *S. cerevisiae* and *A. thaliana*.[41] Pep12p and Vam3p, in addition to the R-SNARE, Vti1p, are critical elements in the sorting of vacuolar proteins. Mutants of each of these proteins cause improper vacuole development.[50-52] Pep12p and Vti1p mutants have reduced viability [51;52], whereas Vam3p mutants grow in a similar fashion to wild type (Figure 6.4).[50] Pep12p and Vam3p exhibit redundancy and have been shown to be capable of compensating for each other's loss.[6;50] Vti1p is involved in two transport steps, interacting with Pep12p in Golgi to prevacuolar transport and with Sed5p (syntaxin-5 homologue) in trafficking to the *cis*-Golgi.[8;52] It has been demonstrated that mouse syntaxin-7 can function as a replacement for deleted Pep12p and Vam3p in homotypic vacuole fusion.[45] Interestingly, syntaxin-7 is upregulated during melanogenesis in B16 melanoma cells, suggesting it may be important for the development of melanosomes.[44] A human homologue of Vti1p, hVti1, has also been identified and can, similarly, serve as a substitute in the two yeast transport steps mediated by this R-SNARE.[53]

The homotypic fusion of yeast vacuoles involves the R-SNAREs Nyv1p, Vti1p and Ykt6p, which are localized to the vacuolar membrane. These R-SNAREs are incorporated into *cis*-SNARE complexes with Q-SNAREs Vam3p and Vam7p (SNAP-25 homologue), on the vacuole membrane.[54] In contrast to the model proposed for synaptic vesicle fusion (see Introduction: section 2.2), ATP dependent

disassembly of *cis*-SNARE complexes mediated by Sec17p (SNAP) and Sec18p (NSF) has been shown to be a prerequisite for subsequent fusion.[54;55] Following this disassembly, Vam7p (a soluble SNARE) is released from *cis*-SNARE complexes and incorporated into *trans*-SNARE complexes resulting in downstream fusion (Figure 6.4).[56] Several other molecules have been identified in yeast vacuole fusion, including Ypt7p (Rab) and its recruited proteins ("HOPS" complex of the vacuolar sorting (VPS) family), phosphatidyl inositides, calmodulin and subunits of the vacuolar H^+ ATPase.[54] The above mentioned studies have suggested that granule biogenesis and granule-granule fusions may involve a number of homologues for these yeast proteins. For example, over 40 VPS family members have been identified in yeast. Mammalian homologues of this family have recently been shown to bind to syntaxin-7.[57]

A proteomic approach may help identify several candidates involved in these processes. The analysis of latex-bead containing phagosomes from macrophages and PMNs by such methods has been utilized to identify a large number of proteins recruited to the phagosome during its formation and maturation.[58] A similar approach may provide insights into granule trafficking. The separation of contaminating lumenal proteins from granule membrane proteins may prove to be technically challenging for such experiments. However, digestion of granule membrane proteins with an array of proteases may succeed in separating these components and reveal the nature of the molecules recruited to the granule membranes during different developmental stages and/or following agonist-induced degranulation.

Since granules from eosinophils and PMNs are not docked to the plasma membrane, unlike synaptic vesicles and cortical granules from sea urchin eggs, it is unlikely that an *in vitro* system can be designed to examine fusion events with the plasma membrane in these cells. However, it is plausible that granule-granule fusions may be examined in a similar manner to cell free assays described for yeast or proteoliposomes. A large scale test of pharmacological inhibitors using such a system may reveal candidate proteins involved in granule-granule fusions, which could then be explored in more detail. This approach could utilize either labeling of lipids with fluorophores for resonance imaging during fusion, as previously used by Rothman and colleagues.[59;60] Alternatively, the yeast vacuole fusion system is a highly quantitative assay based on the generation of a functional enzyme that occurs only following membrane fusion (Figure 6.5).[54;61] While the generation of deletion mutants (used in the yeast system) for human granulocytes is impractical, transfection of fluorescently-labeled granule membrane using retroviral vectors and/or protein delivery methods is achievable and may permit for such an *in vitro* approach. It has been previously shown that delivery of exogenous proteins into human eosinophils was successful using a commercially available protein conjugate (Chariot, Active Motif, Carlsbad, CA).[62] Preliminary experiments in our laboratory have shown that ~60% of human eosinophils are positively labeled with PE-labeled antibodies conjugated to Chariot according to the manufacturer's protocol (Figure B.1, Appendix B).

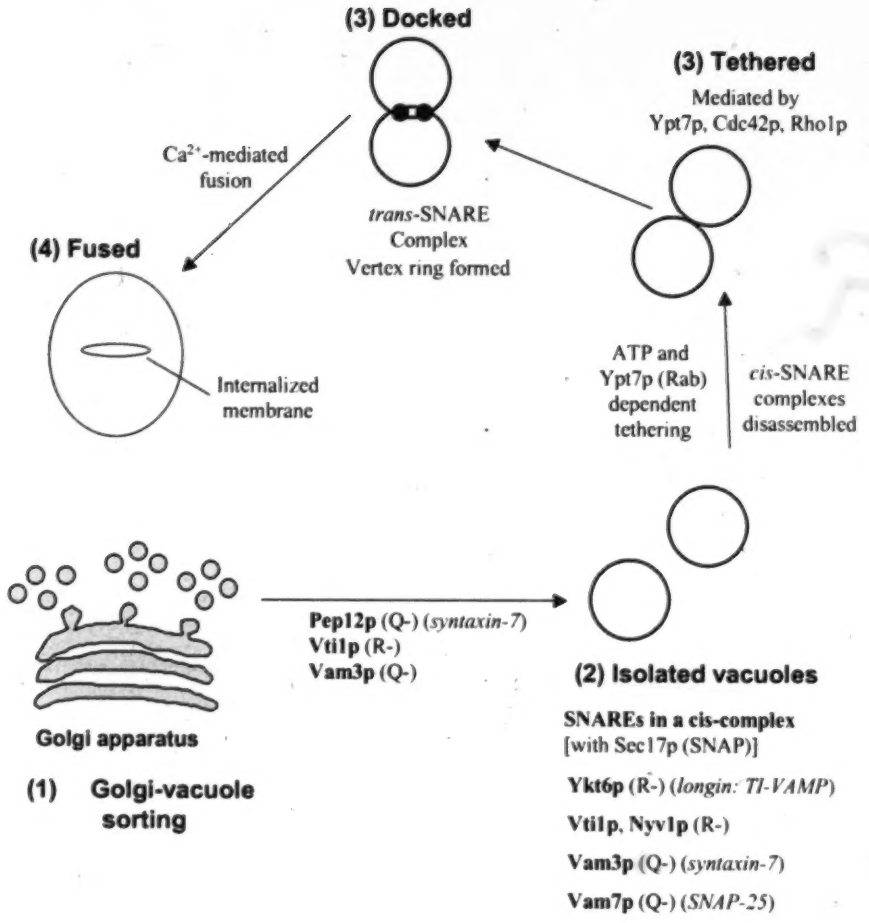

Figure 6.4: Simplified overview of yeast vacuole fusion. Q-SNAREs (Q-), pep12p and Vam3p (both which exhibit homology to human syntaxin-7: brackets) are critical proteins for the delivery of lumenal vacuole proteins from the Golgi apparatus. The R-SNARE (R-), Vti1p, is also critical in this process. Deletion of pep12p, Vti1p or Vam3p cause impaired maturation of yeast vacuoles. R-SNAREs localized to the vacuole include, Ykt6p, Nyv1p and Vti1p. Ykt6p is a longin family member, similar to tetanus-insensitive VAMP (TI-VAMP). Vacuole Q-SNAREs include Vam3p and VAm7p (homologous to human SNAP-25). R- and Q-SNAREs on the vacuole are assembled into cis-SNARE complexes with Sec17p (SNAP) prior to fusion. Sec18 (NSF) disassembles cis-SNARE complexes in an ATP-dependent manner which is accompanied by Ypt7p (Rab GTPase)-mediated tethering of the vacuoles. This tethering event is followed by other tethering events mediated by the GTPases, Cdc42 and Rho1p. The formation of *trans*-SNARE complexes are associated with the formation of a fusion pore which expands in a radial fashion, forming a vertex ring. Ca^{2+}-dependent fusion of the two vacuoles occurs, which results in the internalization of the membrane inside the vertex ring. Modified from: *Wickner W. 2002. EMBO J. 21(6) 1241-47.*

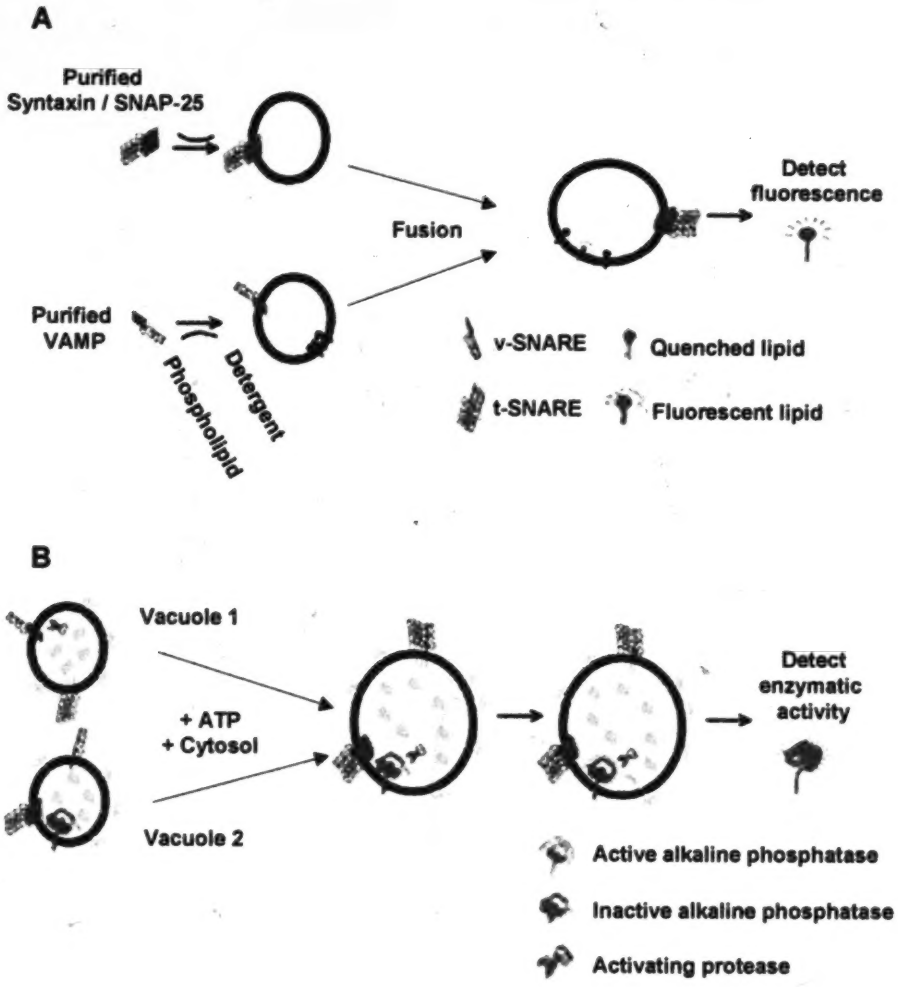

Figure 6.5: In vitro fusion assays (*A*) In vitro liposome fusion assay. Purified recombinant SNAREs are reconstituted into artificial liposomes. The liposome with the v-SNARE contains a quenched fluorescent lipid that is dequenched upon fusion. (*B*) In vitro vacuole fusion assay. Vacuoles from two different yeast strains are isolated. One contains unprocessed alkaline phosphatase; the other has no alkaline phosphatase. Upon fusion, alkaline phosphatase activity is measured.
Modified from: Ungar et al. 2003. Annu Rev Cell Dev Biol. 19:493-517.

Targeting of TI-VAMP to lysosomes and the role of the sorting adaptor, AP-3

The sorting protein adaptor, AP-3, has been shown to be critical for correct localization of lysosome membrane proteins such as LAMP-1,-2 and CD63 (Figure 6.6).[63-65] *Mocha* or *pearl* mice, which have mutated subunits of AP-3, exhibit storage deficiencies in lysosome compartments such as platelet dense granules and melanosomes.[66;67] AP-3 is also involved in the budding of synaptic microvesicles from endosomes during nerve development.[68] However, it has been pointed out that since *mocha* mice exhibit no detectable abnormalities in nerve terminals, AP-3 is unlikely to be required for the biogenesis of all synaptic vesicles.[69] *Mocha* and *pearl* mice are models for the autosomal recessive inherited disease, Hermansky Pudlak syndrome (HPS) found in populations worldwide. Patients with HPS exhibit storage pool deficiency of platelet dense granules, hypopigmentation and accumulation of ceroid pigments in lyososomes. Considerable morbidity and mortality from HPS are caused by fibrotic lung disease, granulomatis colitis and prolonged bleeding.[70]

Recently, Martinez-Arca *et al* (2003) demonstrated that the δ subunit of AP-3 interacted directly with the *longin* domain of TI-VAMP. Full length wild type TI-VAMP was re-directed from LYS to EE when expresed in *mocha* cells lacking AP-3δ. Deletion of the *longin* domain caused a similar effect on TI-VAMP localization supporting the hypothesis that the interaction of TI-VAMP with AP-3 is necessary for its proper targeting to LYS.[71] It is not yet determined if TI-VAMP, itself, is necessary for the delivery of lumenal and/or lysosomal membrane proteins, although this study suggests a potential role for this R-SNARE in this pathway. Clark *et al* (2003) examined CTLs obtained from a patient with HPS type 2 (HPS2) for their ability to

release stored granule proteins and to eliminate target cells. CTLs were shown to lack β3A, γ and μ3A subunits of AP-3 and, as anticipated, CD63 was re-directed from granules to the plasma membrane. Interestingly, perforin and granzymes were correctly localized to granules in AP-3-deficient cells. HPS2 CTL granules failed to polarize to the cell periphery upon contact with target cells and was correlated with impaired killing efficiency.[72] In a separate study, W2 cells (B-lymphoblastoid cell line derived from a patient with HPS-2 who carries a single nucleotide substitution in one allele of the *β3A* gene and a 63 bp deletion in the other gene allele) were shown to exhibit impaired antigen presentation mediated by CD1b. This defect was found to correlate with an inability for CD1b to access the LYS.[73] Together, these observations argue in favor of a role for AP-3 in LYS and lytic granule trafficking and fusion events, which may also include granules from other granulocyte types. It has not been determined whether AP-3, itself, is directly involved in membrane fusion events and/or exocytosis. It is plausible that LYS and lytic granules from AP-3 deficient cells in these studies lack membrane transport proteins such as TI-VAMP and other SNAREs which are more directly responsible for these effects.

The expression and role of AP-3 in granule biogenesis and/or TI-VAMP-mediated trafficking of granules in eosinophils and PMNs has not yet been examined. Galli and colleagues reported that overexpression of the *longin* domain alone impaired exocytotic events during nerve development and arrested axon and dendrite elongation.[9;10] However, the mechanisms for this have not been precisely defined. It has been shown that truncated recombinant TI-VAMP, lacking the longin domain, partners more readily with Q-SNAREs, such as syntaxin-1/SNAP-25 and syntaxin-

4/SNAP-23, than full length TI-VAMP. [9;71] Thus, the presence of the *longin* domain, in the absence of other regulatory proteins, causes structural inhibition of TI-VAMP for assembly into SNARE complexes. Similar binding properties have been previously demonstrated for recombinant syntaxin-1 *in vitro*. The N-terminus Habc domain of syntaxin-1 is folded over the SNARE-binding motif resulting in a "closed" conformation unavailable for SNARE assembly.[74] An "open" conformation of syntaxin is postulated to be regulated by the munc-18 (mammalian homologue of unc-18) family of syntaxin-binding proteins, which have been shown to be essential for membrane fusion (see section IV).[74;75] It is possible that the inhibitory effect of the *longin* domain, *in vivo*, may be due to a similar interaction with a yet to be identified regulatory protein(s). Based on the observation that the *longin* domain interacts with AP-3 [71], this may suggest a role for AP-3 in exocytosis. Further studies are required to determine if the *longin* domain can function as an inhibitor of exocytosis in eosinophils and PMNs and to determine its mechanism of action in this respect.

Although granulocytes are known to have several other effector roles other than secretion (Chapter 1, section 1.5), it is postulated that much of the tissue damage associated with their recruitment is due to the release of pre-formed mediators. The availability of *mocha* and *pearl* mice will allow for the design of future studies to examine the role of AP-3 in the trafficking of TI-VAMP$^+$ granules/vesicles and exocytosis of granulocytes, *in vivo*. These experiments would also provide a potential opportunity to determine the effect of granlocytes defective in granule stores and/or granule secretion on pulmonary inflammation. Both *Mocha* and *pearl* mice are derived from a C57Bl/6J background [66;67], the latter of which has been utilized as a model for

allergen-induced airway inflammation.[76;77] Thus it is possible to design experiments to address the *in vivo* roles of eosinophils and PMNs lacking AP-3 in adoptive transfer experiments. Although eosinophils are not abundant in the circulation of wild type mice, much higher numbers of these cells could be obtained by breeding the AP-3 deficient lines with IL-5 transgenic mice (also available with a C57Bl/6J background).[76]

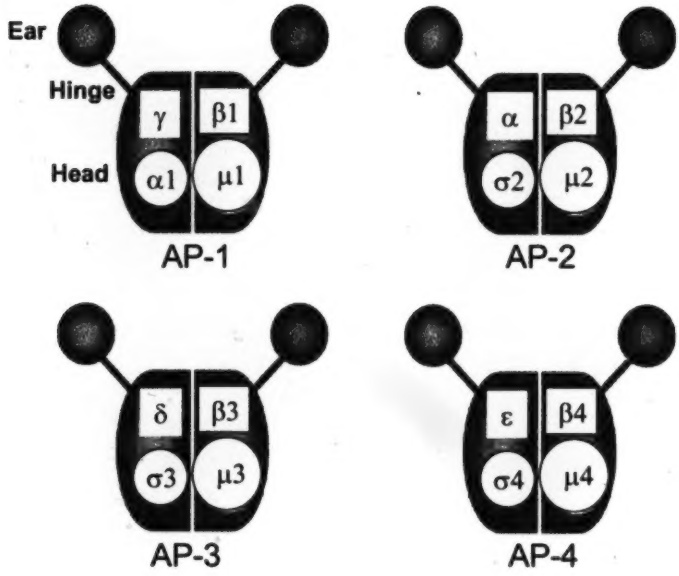

Figure 6.6: Diagrams of the four adaptor protein (AP) complexes. All four complexes consist of two large subunits: a b subunit and a more divergent subunit, either g, a, d, or e ; a medium (m) subunit; and a small (s) subunit. The carboxy-terminal domains of the two large subunits project as 'ears', connected to the 'head' of the complex by flexible hinges. Yeast two-hybrid experiments have shown that the g /a/d /e subunits interact with the s subunits, that the b subunits interact with the m subunits and that the two large subunits interact with each other. b units are important for clathrin binding. The m and b subunits have been implicated in cargo selection and have been reported to bind to sorting signals contained in the cytoplasmic tails of transmembrane proteins. AP-1 and AP-2 are components of clathrin-coated vesicles associated with the trans-Golgi network (TGN) and plasma membrane, respectively. AP-3 is implicated in sorting of lysosome membrane proteins such as LAMP-1, -2 and –3 (CD63). AP-4 has been reported to localize to the TGN and may be involved in TGN to the endosome-lysosome network, similar to AP-3.
Modified from: Robinson,MS & Bonifacino, JS. 2001. Curr. Opin. Cell. Biol. 13(4) 444-53.

III. Implications for piecemeal degranulation and secretory vesicle trafficking

Based on previous studies, it was anticipated that Ca^{2+} and GTPγS activation of permeabilized eosinophils and PMNs would cause the release of multiple granule compartments.[2-5] Consistent with these previous findings, we observed that addition of Ca^{2+} (pCa5) and GTPγS caused a rapid (within 10 min) release of both small (SG and TG) and large (AG) granule mediators from human PMNs (Chapter 5). We have not yet examined exocytosis of SV specifically from human PMNs, although this may be addressed in future experiments by determining the alkaline phosphatase activity in cell supernatants.[78;79] The release of SV from eosinophils is, however, more difficult to assess experimentally, as no conclusive marker has been identified for this compartment.

The R-SNAREs, VAMP-2, TI-VAMP and VAMP-8 were all detected in membrane-enriched fractions from eosinophils and PMNs and are, thus, potential candidates involved in SV trafficking. It is postulated that a portion of secreted EPO is mediated by VAMP-2^+ SV in SLO-permeabilized eosinophils, which correlated with the partial inhibitory effect of VAMP-2 mAb. Further studies are needed to determine the localization and role of these R-SNAREs in SV trafficking. A study by Bandiera-Melo *et al* (2002) reported that IL-4, but not RANTES, was released from eosinophils following stimulation with IL-16, RANTES or eotaxin.[80] This observation suggests that there is a potential for heterogeneity of stored mediators in the SV pool and, by inference, SNARE isoforms. It will also be of interest to examine the role of the reported SNARE isoforms in cells stimulated with other agonists (eg:

cytokine/chemokine receptors). It is likely these experiments will need to be performed in intact cells since the loss of cytoplasmic signaling proteins in permeabilized cells will impair the responsiveness to surface receptor ligands. It is anticipated that the development of transfection and/or protein delivery methods, as discussed above in section II, will be instrumental for such future experiments.

In resting eosinophils, similar to PMNs, SV are distributed throughout the cytoplasm and are readily mobilized and exocytosed following cell activation.[29;81;82] It has been shown that PMN SV are not re-populated following their secretion,[83;84] an observation not yet established for eosinophils. RANTES mRNA and protein have been reported to be re-synthesized following its release from eosinophils, which coincided with the appearance of punctate cytoplasmic staining for this chemokine.[29;85] However, it is not known if these compartments are similar to the stored SV pool in unstimulated cells or may represent a new post-Golgi-derived vesicle population. SV from both cell types contain albumin, indicating an endocytic origin.[86;87] It may be hypothesized that SNAREs localized to SV are involved in endocytic recycling. Candidates for this include VAMP-2, VAMP-3 and VAMP-8, localized previously to endosomes in other cells. [88] Studies have not supported a role for TI-VAMP in endocytosis or homotypic fusion events of EE.[14;15] However, exocytosis of TI-VAMP vesicles has been observed in epithelial cells [36] and developing neurons [9;10] indicating that TI-VAMP may be a candidate R-SNARE for SV exocytosis. As discussed in section II, it is also possible that TI-VAMP is involved in transport from the TGN to granules.[14]

"Budding" of eosinophil CGs has been suggested to be a mechanism for PMD. According to this hypothesis, secretory vesicles derived from CG are postulated to shuttle CG cargo to the cell periphery for mediator release.[89;90] A modified version of this model has been suggested by Moqbel and colleagues (2002). According to this proposed scheme, SV serve as a rapidly mobilizable pool for exocytosis of stored mediators. Following their exocytosis, SVs are recycled and may then fuse with CG to transport CG cargo to the surface. It is postulated that waves of these exocytotic and recycling events occur as the driving mechanism for PMD (Figure 1.4B, Chapter 1).[29;34]

It has not been clearly demonstrated whether granule-vesicle fusions actually occur during PMD, nor has there been any confirmation of CG budding by methods other than electron microscopy. If budding occurs as a mechanism for PMD, it is anticipated that the vesicles derived from membrane evaginations would contain similar membrane proteins to CG. In addition, it seems plausible that these vesicles are likely to be distinct from the cytoplasmic pool of SV. For example, the stored cytoplasmic SV pool contains albumin, whereas CG do not. In addition, the stored SV pool does not appear to express CD63, a marker of CG. It is anticipated that if budding occurs, the secretory vesicles derived from CG would contain similar membrane proteins. From our studies, this may suggest that vesicles expressing TI-VAMP and/or VAMP-8 may be derived from CG. In contrast, the observation that VAMP-2 is not localized to CG suggests that VAMP-2$^+$ vesicles are unlikely to be derived from CG. It is also evident that a recycling event likely occurs during PMD, since cytoplasmic CG with eroded contents do not appear to be substantially of smaller size than "intact" CG

by electron microscopy.[89] Thus, a mechanism must be in place to maintain the overall surface area of the CG membrane, possibly through endocytic recycling. In both models of budding and recycling, membrane evaginations from CG would also undoubtedly require the recruitment of coat and adaptor proteins involved in membrane budding.[69;91] If the recycling model is a mechanism for PMD, it would also be expected that CG would acquire markers of EE or recycling endosomes (eg: Rab5 and its effector, EEA1 and/or Rab11).[92] These predictions remain to be determined experimentally and may shed light on the mechanism of PMD in these cells. As mentioned in section II, a proteomic screening approach to examine changes in CG membrane proteins during activation may be useful in the identification of such proteins. In addition, labeling of the identified membrane markers by immunogold electron microscopy will be useful to examine membrane budding and/or recycling events.

An alternative explanation for eroded cytoplasmic CG in eosinophils may be suggested from synaptic vesicle cycle. In neurons, two distinct modes of vesicle recycling have been identified. A slow mode (tens of seconds) which occurs via clathrin-coated vesicles and a rapid mode (milliseconds) which is a quick reversal of the exocytic event without intermixing of the plasma (presynaptic) membrane. This fast recycling has been termed "kiss-and-run" fusion.[93-95] The number of fast recycling events has been shown to increase with elevated intracellular calcium in neurons and chromaffin cells.[96;97] In chromaffin cells it was shown that full discharge of granule contents could occur via "kiss-and-run". Thus, complete fusion with the plasma membrane is not essential for granule content release.[96] The mechanism of

recycling for these rapid remains unclear. Holroyd *et al* (2002) demonstrated that vesicles retrieved along this rapid pathway were associated with dynamin and peptide inhibitors of dynamin blocked their recycling.[98] Their findings suggest that kiss-and-run endocytosis is unlikely to be mediated by a reversal of SNARE complex assembly.

It is not known if kiss-and-run type fusion and recycling events occur for CG. However, it is possible that such an event may be, at least in part, responsible for the presence of eroded cytoplasmic CG from tissue eosinophils at sites of inflammation. Lindau and colleagues have demonstrated that fusion pores in both eosinophils and PMNs exhibit a conductance similar to an ion channel. This suggests the fusion pore may be formed by a membrane spanning protein or protein complex.[99;100] SNAREs represent only one candidate family implicated in membrane fusion, and it is still unclear if they are directly responsible for pore formation (Chapter 1, section 2.3). In eosinophils and PMNs, fusion pores appear reversible, at least for a brief period following their formation [99;101]. Similar reversibility and conductance properties have been reported for larger mast cell granules.[102;103] Collectively, these observations suggest that transient and reversible fusions, like kiss-and-run, may occur in these cells.

IV. Regulators of SNARE assembly and other exocytotic proteins

Several proteins have been identified to bind and potentially regulate SNARE localization and or their assembly. While it is beyond the scope of this project to discuss all of these regulators, a brief discussion of the families of proteins for which

we have preliminary experimental data is hereby described. Current studies on these molecules continue in our lab to determine their role in SNARE localization and/or assembly as well as mediator secretion.

Sec1/Munc18 (SM) proteins: A family of conserved syntaxin-binding proteins, SM proteins, has been directly implicated in regulation of SNARE complex assembly. SM proteins were initially identified as critical vesicular transport proteins in yeast (Sec1) and *C. elegans* (unc-18).[104;105] Three mammalian homologues of unc-18 (Munc-18) have been identified: Munc18-1(or -a), -2(b) and –3(c).[106] Munc18-c is the most broadly expressed isoform, and has been identified in non-neuronal tissues.[107-112] SM proteins have been demonstrated to be essential for secretion. Deletion mutants in *C. elegans* (unc18) [104], *Drosophila* (Rop) [113;114] and mice (Munc18-a)[115] are all paralyzed and devoid of secretion from neuronal tissues.

SM proteins selectively bind to a "closed" conformation of syntaxin. In this conformation, syntaxin amino terminal region is folded over its SNARE-binding region. Thus, when bound to Munc18, syntaxin has an impaired ability to associate with other SNAREs and form docking complexes (Figure 6.7).[74;116;117] Similar to the reported promiscuous interaction of SNARE isoforms, Munc18 isoforms can bind multiple syntaxins, at least *in vitro.* Munc18-a and b exhibit similar binding to syntaxin-1A, 2 and 3 [118-120] while Munc18-c is capable of interacting with syntaxin-2 and 4 [118]. These observations lend further support to the notion that the specificity of granule/vesicle docking is unlikely to be determined by a single family of proteins, but is the result of coordinated interactions of multimeric signaling complexes.

Preliminary data generated in our laboratory indicated human eosinophils expressed mRNA and protein for Munc-18c. Munc-18c is localized to membrane-enriched fractions and, to a lesser extent, EPO-enriched granules.(Figure B.2, Appendix B). Houng *et al* (2003) recently reported that human platelets expressed all three mammalian SM isoforms, munc18-a, b and c, which were detected in membrane and cytosolic fractions. In their study, administration of peptides or neutralizing antibodies that interfere with the assembly of Munc18-c-syntaxin complexes resulted in a potentiation of Ca^{2+}-induced secretion. These observations support the assumption that the disassembly of Munc18-syntaxin complex is associated with mediator release in platelets.[112] SM proteins have additionally been critically implicated in mediator secretion from mast cells.[121]

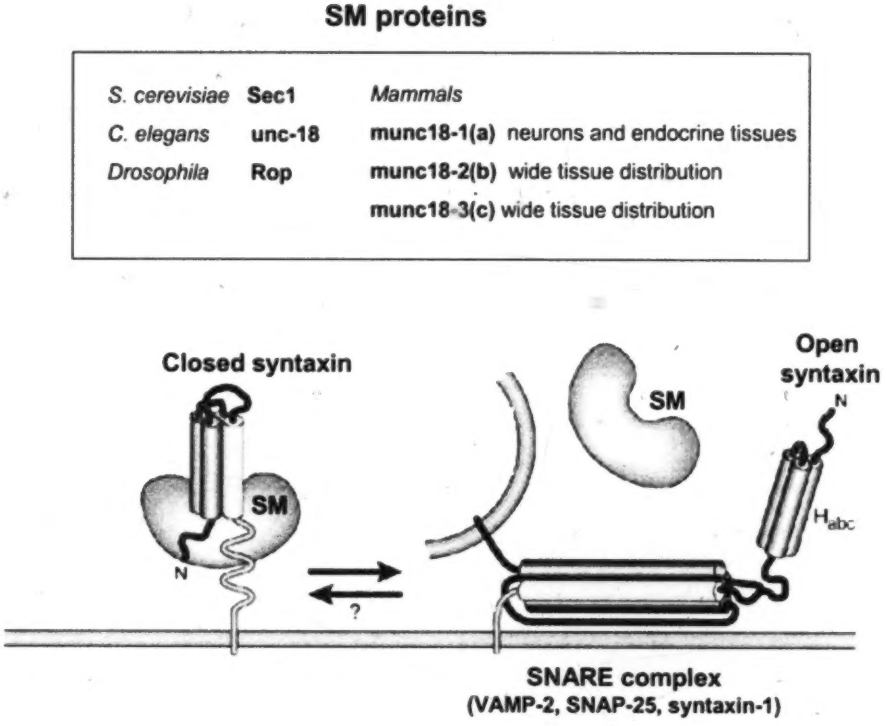

Figure 6.7: Model of Sec1/Munc-18 (SM) protein function in SNARE assembly and exocytosis

(Left) Syntaxin-1 is in a "closed" conformation when bound to Munc18-a and is not available for assembly into SNARE complexes. In this closed conformation, the Habc amino terminal region of syntaxin-1 is folded over its SNARE binding motif. SM family proteins are essential for secretion , and deletion of SM proteins in C. elegans, Drosophila and mice (Munc18-a deletion) cause severely impaired nervous secretion and paralysis. *(Right)* It is postulated SM proteins are critical for regulating SNARE assembly. The release of Munc18-a from syntaxin-1 may be mediated by phosphorylation events mediated byeither protein kinase-c (PKC) isoforms or cyclin-dependent kinase-5 (cdk-5). Phosphorylated Munc18-a has a reduced affinity for syntaxin-1. An "open" linear form of syntaxin-1 assembles into SNARE complexes with VAMP-2 and SNAP-25.

Modified from: Rizo J and Sudhof TC. 2002. Nature Rev. Neurosci. **3** 641-653

Regulation of the SM-syntaxin complex and SNARE partnering

The activation of protein kinase C (PKC) by diacylglycerol (DAG)- and Ca^{2+}-mediated activation is regarded as a key triggering step in exocytosis from a variety of cell types. Bates *et al* (1993) have reported the expression of conventional, novel and atypical isoforms of PKC in eosinophils.[122] A role for PKC in mediator secretion from human eosinophils is supported by observations of both PMA [123] and allergen-induced mediator release from eosinophils.[124] [125]Similarly, studies in PMNs,[126-128], mast cells,[129;130] lymphocytes[131] and platelets [132;133] suggest that PKCs may be essential in regulating degranulation in hematopoietic cells. It is postulated that an important effector function of PKCs is the regulation of SNARE partnering with other SNAREs and/or SNARE-associated accessory proteins. SNAP-25, VAMP-2 and SM proteins are all phosphorylated by PKC, *in vitro*. [134-137] PKC-mediated phosphorylation of SNAREs is reported to reduce their affinity for SNARE-SNARE partnering.[132;134] Similarly, PKC-mediated phosphorylation of SM proteins reduced their affinity for syntaxins *in vitro*.[111;136] An attractive model for exocytosis is that stimulus-coupled Ca^{2+}-mediated activation of PKC leads to the phosphorylation of Munc18 and/or SNAREs, resulting in an "open" syntaxin conformation for SNARE assembly.[136]

Another candidate for regulation of SM-syntaxin complex is cyclin-dependent kinase-5 (cdk5). Cdk5-mediated phosphorylation of Munc18-a has been reported to occure in the absence of a Ca^{2+} flux in association with mediator release from neuroendocrine cells. Munc-18-a when phosphorylated by cdk-5 *in vitro*, has a reduced affinity for syntaxin-1 similar to the effect mediated by PKC. The Thr^{574} cdk-

5 recognition site is highly conserved in SM proteins from yeast to mammals.[138] Cdk-5 has also been shown to be critical for synaptic vesicle endocytosis by phosphorylating "dephosphin" proteins involved in recycling events. These include dynamin I, amphiphysin I and II and synaptojanin.[139;140] The expression of dominant-negative cdk-5 has been shown to impair synaptic vesicle recycling [140] and secretion of insulin from pancreatic β-cells.[141] In addition, pharmacological inhibitors of cyclin-dependent kinases, olomoucine and roscovitine, have been shown to impair exocytosis from chromaffin cells[138] and pancreatic β-cells[141] and synaptic vesicle recycling.[140]

The role of cdk-5 has not been well established beyond the nervous system and endocrine tissues. Recent studies have revealed that active cdk-5 is expressed in non-neuronal tissues. Elevated cdk-5 has been reported to increase during myogenesis in mouse C2 cells [142] and is expressed in lens epithelial cells in rats.[143] In addition, the expression of cdk-5 and its upstream activator, p35, have been identified in differentiating monocytes from the leukemic cell line, HL-60.[144] Our laboratory has demonstrated that cdk-5 is expressed in resting human peripheral blood eosinophils. Preliminary studies indicated that the pharmacological inhibitor, roscovitine, impaired EPO release from eosinophils activated with calcium ionophore. This effect appeared to be specific for exocytosis, as PMA-induced release of superoxide was not affected by roscovitine treatment (*S.O. Odemuyiwa: unpublished observations*). The role of cdk-5 in the phosphorylation of SM proteins and other potential targets is currently being investigated in human eosinophils.

Monomeric GTPases: Rabs and others

Rabs comprise a diverse family (>40 isoforms) of the Ras-related monomeric G proteins. Several studies have supported that Rab isoforms localize to membranes of distinct intracellular compartments, lending credit to the notion that this family of proteins may be particular important in directing distinct granule/vesicle populations to their appropriate destinations.[145-147] A wide variety of Rab effector proteins have been identified, and are involved in a diverse number of roles including Golgi vesicle budding, recruitment of cytoskeletal proteins for organelle/vesicle movement and protein-protein tethering at granule/vesicle docking.[146;148-150] Recent studies have implicated a number of Rab isoforms in exocytosis and SNARE assembly in hematopoietic cells.

CTLs derived from Rab27 deficient mice exhibited impaired release of granule proteins, granzyme A and hexosaminidase, and did not efficiently kill target cells.[150] In a separate study, Torii *et al* (2004) recently demonstrated that the Rab27 effector, granuphilin, directly interacts with the H3 domain of syntaxin-1a in pancreatic β-cells. The overexpression of granuphilin mutants that were incapable of binding either syntaxin-1a or Rab27 were shown to cause impaired granule recruitment to the plasma membrane.[151] These observations support that Rab27 is involved in tethering granules to the plasma membrane. A similar tethering and/or docking role for Rab4 is suggested from the observation that recombinant Rab4 directly interacted with syntaxin-4, *in vitro*.[152] In platelets, Rab4 was localized to alpha granule and the addition of a dominant-negative Rab4, incapable of hydrolyzing GTP, was shown to impair mediator release from permeabilized cells.[153] Preliminary data generated in

our laboratory have shown both Rab4 and Rab27 are expressed in human eosinophils. Rab27 was detected in EPO-enriched granules, whereas only a minor immunoreactive band was detected for Rab4 in these fractions.(Figure B.3, Appendix B). In RBL-2H3 cells, Rab3D was precipitated with an unidentified Ser/Thr kinase of ~130 kDa (termed Rak3D) which was capable of phosphorylating syntaxin-4 *in vitro*.[154;154] In addition, a potential role for Rab6 in exocytosis may be suggested from the observation that this isoform was phosphorylated in a PKC-dependent manner following thrombin-induced activation of platelets.[133]

As discussed in section II, it is postulated that biogenesis and exocytosis of CG and AG from eosinophils and PMNs, respectively, may utilize conserved protein machinery identified for yeast vacuole fusion. In addition, it is anticipated that exocytosis of these compartments shares several similarities with the exocytosis of lysosomes. In yeast vacuole fusion, a sequential recruitment of GTPases has been shown to be involved in docking interactions. These include the Rab Ypt7p and Ypt7-recruited effectors, which are followed by the Rho family GTPases, Rho1p and Cdc42p.[155;156] In mammalian cells, Rab7 and Rab9 have been shown to be involved in transport from the EE to the LE/LYS and recycling of the M6PR from endosomes, respectively.[157-159] The expression and role of Rab7 and Rab9 in granulocyte exocytosis has not yet been examined.

Cdc42 and Rac2 (both Rho family members) have been shown to be involved in chemotactic signaling and superoxide production from PMNs and eosinophils.[160-163] Abdel-Latif *et al* (2004) showed that Rac2-deficient PMNs derived from mouse bone marrow exhibited impaired release of MPO. Interestingly, the release of

lactoferrin and MMP-9 was similar in wild type and Rac2-deficient cells suggesting that Rac2 has a selective role in AG exocytosis.[164] It remains to be determined if Rac2 is predominantly involved in signaling events required for granule mobilization or docking of granules with the plasma membrane.

V. SNARE isoforms involved in granule/vesicle trafficking from other hematopoeitic cells

A number of recent studies, as well as observations from our laboratory, have demonstrated a critical role for SNAREs in granule/vesicle trafficking in other hematopoietic cell types. Below is a brief description of isoforms that have been implicated in granule/vesicle exocytosis and docking with the plasma membrane.

R-SNAREs

VAMP-2, 3, -8 and TI-VAMP have all been reported to localize to granules and/or SV in the rat basophilic cell line, RBL-2H3. [165;166] The observation that overexpressed GFP-TI-VAMP colocalized with the granule marker, CD63, may suggest it plays a role in exocytosis, similar to eosinophils and PMNs.[167] However, no studies to date have examined the functional role of R-SNAREs in exocytosis from these cells. Similarly, tissue mast cells have not been studied in detail in respect to R-SNARE function, although VAMP-2 was shown to exhibit a punctate intracellular staining pattern in rat peritoneal mast cells (RPMC).[168] Preliminary date from our laboratory has indicated that, in addition to eosinophils and PMNs, other cell types with LYS-like storage granules express TI-VAMP. These include the rat basophilic

cell line, KU812, human periperal blood basophils, antigen-specific cytotoxic lymphocyte (CTL) clones and the human NK cell line, YT Indy.[169;170] Each of these cell types also express protein for VAMP-2, -3 and –8. Further studies are currently underway to determine their functional role in the release of stored mediators.

Studies by separate groups have indicated that VAMP-3 [171-173] and 8, [173] but not VAMP-2, [171;174;175] are expressed in human platelets. Electron microscopy studies by Feng *et al* (2002) [172] indicate that VAMP-3 is predominantly localized to alpha-granules, although not all cytoplasmic granules exhibited VAMP-3 immunoreactivity. Functional analyses of secretion in SLO-permeabilized platelets utilizing neutralizing antibodies[172], TeNT (which cleaves VAMP-3, in addition to VAMP-2) [175] and peptide analogues [173], have indicated that VAMP-3 is involved in mediator release from alpha and/or dense granules. These findings, however, are in contrast with a later report that showed no impaired secretory response of the three platelet granule populations in platelets derived from VAMP-3 deficient mice.[176] The precise localization of platelet VAMP-8 has not been identified. The addition of recombinant VAMP-8 to SLO-permeabilized platelets was reported to impair mediator release from dense granules but not alpha granules, which could suggest it is exclusively involved in secretion of the former granule type.[173]

VAMP-2 and 3 have been implicated in exocytosis of SV following activation of phagocytosis in murine macrophages.[177;178] Hackam *et al* (1998) reported that treatment with TeNT impaired phagocytosis in murine J774 macrophages [177]. Clustering of VAMP-3 was found at the site of phagosome formation and was additionally incorporated into early phagosomes, suggesting its involvement in

phagosomal maturation events.[178] A study by Allen *et al*, however, demonstrated that macrophages derived from VAMP3 deficient mice did not exhibit impaired phagocytosis or phagosome maturation, regardless of the type of receptor engagement or particle load. [179] They have suggested that each of VAMP-2, 3 and 8, all of which have been localized to recycling endosomes [88;180;181], may perform redundant roles and/or compensate for each other in phagocytic processes.

Q-SNAREs

A number of observations have indicated that Q-SNAREs in hematopoeitic cells types are localized to multiple intracellular sites. By means of co-precipitation and *in vitro* experiments, SNAP-23 and syntaxin-4 have both been shown to interact with VAMP-2, -3, -8 and TI-VAMP.[33;71;173;182;183] Syntaxin-4 is also capable of binding to SNAP-25 [111;183] and overexpressed SNAP-23 can function as a replacement for impaired SNAP-25.[184] These observations suggest Q-SNAREs are capable of participating in more than one trafficking step. However, the localization of these Q-SNAREs likely defines the transport steps they participate in. Most, if not all, Q/R-SNARE interactions in hematopoietic cells have been identified by co-precipitation studies. Particularly since recombinant SNAREs bind promiscuously *in vitro* [183;185], it is possible that SNARE-SNARE pairings identified by such methods may have resulted following cell lysis and, therefore, might not be representative of interactions *in vivo*. Therefore, SNARE interactions alone are unlikely to be sufficient to assign a particular SNARE to a given transport step.

Electron microscopy studies of human platelets have shown that SNAP-23, syntaxin-2 and syntaxin-4 are localized to multiple membranes including: storage granules, membrane channels and the plasma membrane.[47;172] Similarly, both SNAP-23 and syntaxin-4 were not exclusively localized plasma membranes in eosinophils and other intracellular sites appeared to include the Golgi apparatus and ER, respectively.[34] As discussed in section I, SNAP-23 has been reported to localize to granules, in addition to the plasma membrane, in PMNs.[35] Similarly, SNAP-23 was reported to re-localize from plasma membranes to granules in RPMC following Ca^{2+} and GTPγS-induced activation. The mobilization of SNAP-23 was postulated to be involved in preparing granules for a fusion ready state and/or required for cytoplasmic granule-granule fusions during compound exocytosis in these cells.[168] Docking partners for macrophage R-SNAREs include syntaxin-2, 3, and 4, all of which have been localized to both plasma membrane and isolated phagosomes.[186]

Several of the above mentioned studies have supported a role for Q-SNARE isoforms in exocytosis. Antibodies directed against SNAP-23 were shown to prevent SNAP-23 relocation from the plasma membrane to granules and impaired secretion of the granule mediator, β-hexosaminidase (β-HEX) in SLO-permeabilized RPMCs.[168] In platelets, antibody-mediated inhibition of syntaxin-4 was reported to impair mediator release from both alpha-granules [175] and lysosomes [47], but not dense granules.[46] However, neutralization by SNAP-23 and syntaxin-2 antibodies impaired the secretion of platelet dense granules and lysosomes in a separate study.[46;47] Syntaxin-2, similar to syntaxin-4, can associate with both SNAP-23 and SNAP-25

[47;183]. These observations suggest that the same Q-SNAREs can function in the release of more than one granule type.

Conclusions

An overlapping set of SNARE isoforms have been identified in vesicle/granule trafficking in hematopoeitic cell types, including eosinophils and PMNs. Our data and the observations from other studies suggest that TI-VAMP and molecules involved in its targeting and trafficking play an important role in exocytosis and, potentially, granule biogenesis. Due to the apparent redundancy in isoforms involved in these secretory processes, the design of inhibitors of SNAREs and/or other exocytotic proteins may prove to be effective agents to impair mediator release from a number of leukocytes. Such inhibitors may prove to be clinically beneficial in the treatment of a number of inflammatory conditions. Further research is required to define more precisely the mechanisms of exocytosis in these cell types and to identify additional candidates for targeted inhibition. As well, the challenging task of specifically targeting such inhibitory agents to particular cells and/or microenvironments will need to be addressed in future studies.

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Materials for subcellular fractionation

Buffer A*

	g or ml (250 ml)
0.25 M sucrose	21.39 g
10 mM HEPES, ph=7.4	2.5 ml of 1M
1mM EGTA, ph=7.0	2.5 ml of 100 mM

Adjust ph to 7.4. Filter thru 0.45 um, store in 50 ml aliquots in –20C

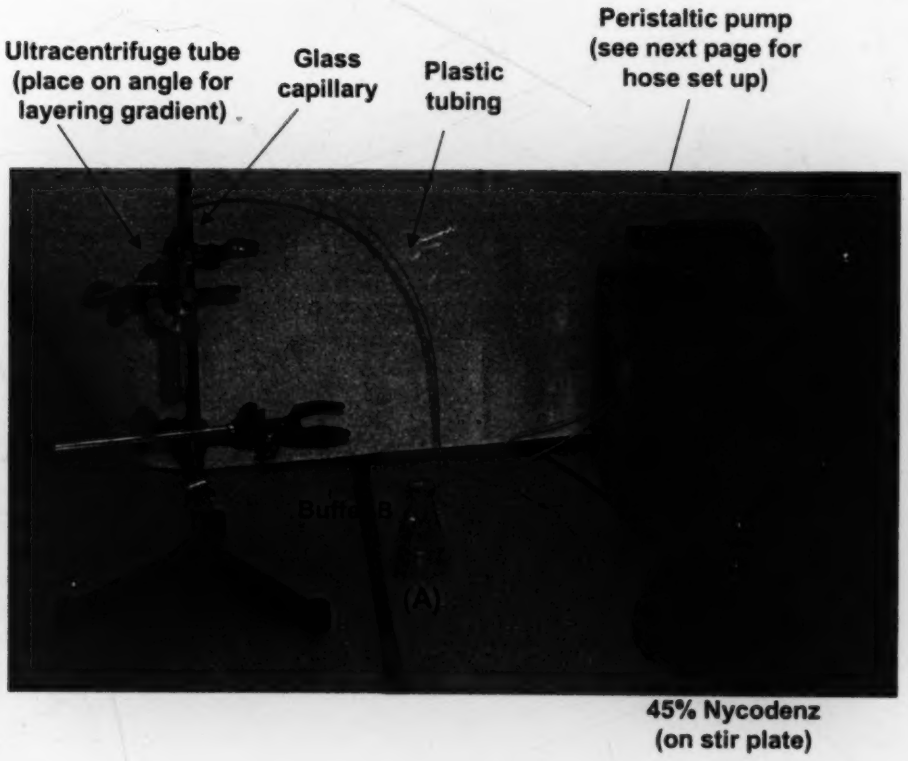
Buffer B (made fresh)

10 ml Buffer A*
+ 2 mM MgCl2 (20 ul of 1M)
+ 1 mM ATP (100 ul of 100 mM)
+ 5 µg/ml protease inhibitors** (5 µg/ml leupeptin, TAME, aprotinin (Sigma)
+ 100 µg/ml PMSF (100 µl of 10 mg/ml stock)

45% Nycodenz

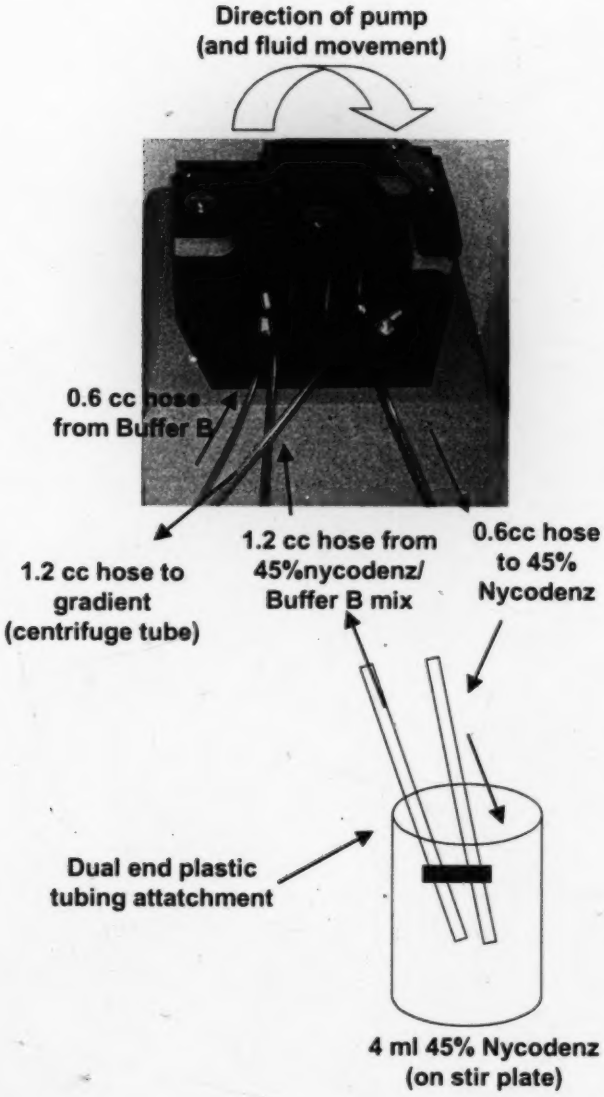
45 g Nycodenz in ~ 75 ml of Buffer A
stir on stirplate ~ 1 hr to dissolve
bring total volume to 100ml with Buffer A
filter thru 0.45 um, store 5ml aliquots in –20C

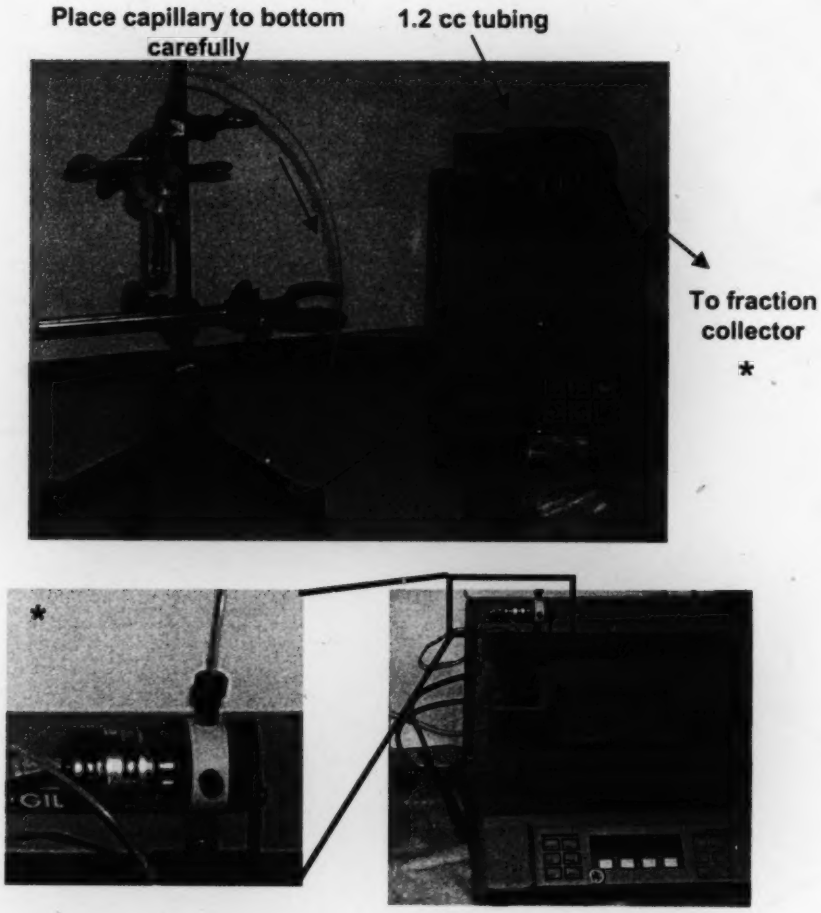
add 5 µg/ml protease inhibitors** prior to use


Ultracentrifuge tube (place on angle for layering gradient)
Glass capillary
Plastic tubing
Peristaltic pump (see next page for hose set up)
Buffer B
(A)
45% Nycodenz (on stir plate)

Preparation of Nycodenz gradient for subcellular fractionation

In an ultracentrifuge tube (Beckman 9/16 X 3 ¾ in: 14X95 mm #344060) place 0.5 ml of 45% Nycodenz into the bottom of the tube (this will serve as a cushion for the heaviest organelles (the granules) to rest on following subsequent ultracentrifugation. Place 4 ml of each 45% Nycodenz and Buffer B into each flat-bottomed tube. Attach 0.6 CC and 1.2 CC plastic hoses with glass capillary (50 ul) according to diagram. Place the end of the hose into Buffer B (A), keep the ends of the hoses (B) **out** of the Nycodenz. Turn on peristaltic pump at +5.00 setting and let run until to Buffer B flow until just ready to leave hose (point *). Turn off the pump and insert hose ends (B) into the 45% Nycodenz. Turn pump back on (note: you cannot turn the pump off at this stage!!!). Layer the gradient on top of the Nycodenz cushion being careful not to lose contact with the interface (this will cause mixing) (note: this takes about 10 min to pour so make sure you have a steady seat). After the gradient is poured, layer the post-nuclear supernatant onto the gradient (note: this is difficult and requires steady hands: transfer pipettes and/or loading the post-nuclear supernatant into a syringe are useful methods).


Direction of pump
(and fluid movement)
0.6 cc hose
from Buffer B
1.2 cc hose to
gradient
(centrifuge tube)
1.2 cc hose from
45%nycodenz/
Buffer B mix
0.6cc hose
to 45%
Nycodenz
Dual end plastic
tubing attatchment
4 ml 45% Nycodenz
(on stir plate)



Collection of subcellular fractions following ultracentrifugation: Set peristaltic pump at +10.0.
Set fraction collector at 24 drops/fraction (eosinophils) or 22 drops/fraction (neutrophils).
Ensure fractions tubes are labeled (note the direction of the collections is different from standard ELISA plates: start : top left, moves down the row, then **up next row from bottom to top**)

Following fraction collection:
Make 1 set of 1:10 diluted fractions (50 ul fraction : 450 ul Buffer A)
Aliquot remaining fractions (3X)
Store fractions at –80C.

SLO permeabilization of eosinophils and neutrophils

Ca^{2+}-EGTA buffer preparation

Materials

Potassium oxalate
1 M CaCl2 (commercially supplied solution)
EGTA
PIPES
50 mL burette

Method

- Prepare 500 ml 0.25 M CaCl2 by diluting 1 M solution 1 : 4 (125 ml 1 M solution + 375 ml dH2O).
- Prepare 500 ml of solution containing ~ 0.2 M EGTA + 40 mM PIPES. Dissolve EGTA by mixing acid form with 4x molar equivalent of NaOH (i.e. 0.4 moles or 20 ml 10 M NaOH in 500 ml). Adjust the pH to neutrality (pH 7).
- Clean all volumetric glassware (burette and pipettes) in detergent (Decon 90) or chromic acid. Rinse extensively in water. Before filling, rinse glassware in appropriate solution.
- Titrate 0.25 M CaCl2 (in burette) with 50 ml buffered chelator after adding 5 ml of 10 M NaOH to keep pH above 10. Approximately 40 ml of CaCl2 will have to be added. Add indicator to the buffered chelator (2.5 ml 1 M potassium oxalate) and titrate until cloudiness persists for 1 min.
- Repeat this titration twice.
- Prepare the CaEGTA stock solution using same pipette and burette to mix 50 ml of buffered chelator (omit the alkali and indicator)with the exact same amount of CaCl2 indicated by the titrations. The total volume should be approximately 90 ml.
- Prepare the EGTA stock solution by adding 40 ml water to 50 ml buffered EGTA.
- Adjust the pH of both solutions to 6.80 and bring their volumes up to exactly 100 ml in volumetric flasks.
- These two solutions may be stored in tightly capped Falcon tubes for many months at -20°C. Use the solutions in proportions shown in Table I to achieve the required level of free Ca2+.

Table I
Recipe for Ca^{2+} buffer solutions

pCa **	[Ca]total [EGTA]total	Volume (ml) CaEGTA	EGTA
8	0.014	0.112	7.888
7	0.124	0.996	7.004
6.75	0.202	1.614	6.386
6.5	0.310	2.481	5.519
6.25	0.444	3.555	4.445
6.0	0.587	4.698	3.302
5.75	0.717	5.736	2.264
5.5	0.819	6.552	1.448
5.25	0.891	7.125	0.875
5	0.938	7.501	0.499

Secretion assay for SLO permeabilized eosinophils and neutrophils with incubation of exogenously added antibodies

Materials

Buffered salt solution (BSS), pH 6.8:

	10X stock (500 ml)
137 mM NaCl	**40** g
2.7 mM KCl	**1** g
2 mM $MgCl_2$	**2** g
20 mM PIPES	**3** g or 10 mL 1 M PIPES

- **Adjust pH of stock to 6.8 with 10 M NaOH (do this slowly if using powdered PIPES, as PIPES takes time to dissolve). Check pH of diluted solution before use.**
- **Dilute solution 1:10 before use (usually make up 100 mL for each assay) and add 1 mg/ml BSA.**

EGTA solution (pH 6.8)
10 x 10^6 cells (10 mL) cell suspension/plate (density 1 x 10^6 cells/ml)
10% CHAPS (for neutrophils) or 10% Triton-X-100 (for eosinophils) (in H_2O)
Streptolysin-O (100 µg/mL)
100 mM GTPγS (Boehringer Mannheim 95-97% pure solution)
Calcium buffers (*p*Ca 5 and 8) (made from protocol on previous page: see **)
Inhibitory antibody (AZIDE FREE)
Isotype control antibody (AZIDE FREE)
Sigma TMB solution (T8540)
V-well 96-well microplates (volume/well = 0.25 mL)
Flat-well 96-well microplates (Falcon)
Remove from fridge:

10% CHAPS (in H_2O) (for neutrophil lysates)
10% TX-100 (in H2O) (for eosinophil lysates)
TMB solution (myeloperoxidase substrate) just before use

Remove from freezer:

100 mM ATP (may be frozen and thawed repeatedly)
Streptolysin-O (100 µg/mL) (**do not repeatedly freeze and thaw**)
Calcium buffers (pCa's can be frozen and thawed repeatedly)
100 mM GTPγS (may be frozen and thawed repeatedly)
Antibody (isotype + test)

Method

- Prepare 100 µM GTPγS by adding 1 µl frozen stock (100 mM) to 0.999 ml BSS buffer. Leave on ice.
- For neutrophil assays prepare 1% CHAPS solution in BSS (50 µl 10% CHAPS + 450 µl BSS). For eosinophils prepare 1% TX-100 similarly. Leave on ice.
- Make up two tubes of pCa 5 and pCa 8 solutions:

- **pCa 5** tube: Add 1.733 ml BSS, 900 µl GTPγS stock, 277 µl pCa 5, and 90 µl ATP (100 mM). Leave on ice.
- **pCa 8** tube: Add 2.633 ml BSS, 277 µl pCa 8, and 90 µl ATP (100 mM). Leave on ice.

- For neutrophils: prepare 0.2 µg/ml SLO by adding 10 µl frozen stock of SLO (100 µg/ml) to 4.995 ml BSS. For eosinophils: prepare 2.0 µg/ml SLO solution. Leave on ice.
- Prepare inhibitory antibody to a final concentration of 2 µg/ml in either 0.2 µg/ml SLO (neutrophils) or 2.0 µg/ml SLO (eosinophils). Leave this on ice.
- Prepare inhibitor-SLO solutions to go into microplate by sequential dilution using the LOGSTEPS program. For example, for VAMP-2 mAb (1 mg/ml stock) :
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Tube #	Antibody	SLO (µl of 0.2 µg/ml)	Ab conc with cells (diluted 2X)
1	138.6 µl of #2	160 µl	0.10 µg/ml
2	138.6 µl of #3	160 µl	0.22 µg/ml
3	138.6 µl of #4	160 µl	0.46 µg/ml
4	138.6 µl of #5	160 µl	1.00 µg/ml
5	138.6 µl of #6	160 µl	2.15 µg/ml
6	138.6 µl of #7	160 µl	4.64 µg/ml
7	4.4 µl of 1 mg/ml anti-VAMP-2 or 8.8 µl of 0.5 mg/ml isotype	435.6 µl or 431.2 µl	10.00 µg/ml

Total volume in each tube = 160 µl except for tube #6 = 440 µl

- Make up isotype control by using same series of antibody dilutions in SLO.

Microplate preparation

While spinning down 5 x 10^6 cells and resuspending these in 5 ml BSS, use a V-welled microtitre plate on ice to pipette following reagents.

- Add 50 µl of appropriate inhibitor-SLO mix into each well except Controls in Column 10, Blanks in Column 11 and Lysed Cells in Column 12.
- For **Columns 10 and 11** on the plate, add 50 µl SLO.
- For **Column 12** on the plate, add 50 µl 1% CHAPS in BSS.
- Resuspend cells in BSS at 1 x 10^6/ml. Do not leave cells in BSS any longer than 5-10 min as they gradually lose their secretory capacity.
- Remove pCa 5 and pCa 8 solutions from ice and allow these to warm at room temperature.
- Remove plate from ice and allow this to warm at room temperature.
- Get a reagent tray and adjust a multichannel pipette to 50 µl/well.
- Chill down some BSS to get it ready to stop the reaction.
- Pour cells into a reagent tray and quickly transfer 50 µl cells to all wells. Mix cells briefly to ensure mixing of SLO and inhibitor with cells. When cells are added to **Column 12** on the plate, make sure that you pipette the mixture up and down 3-4 times to assist in complete solubilization of cells.
- Place microplate into 37°C incubator for 2 min. The cells will be permeabilized in this step, so do not run over time!
- Pour pCa 5 and pCa 8 into different reagent trays.

- Remove microplate from incubator at 37°C at 2 min and transfer 50 µl of **pCa 5** into all wells EXCEPT Columns 11 and 12.
- Transfer 50 µl of **pCa 8** into Columns 11 and 12.
- Incubate for 10 min at 37°C.
- Stop reaction by adding 100 µl ice-cold BSS to each well
- Centrifuge plate for 5 min at 1500 rpm (~350g) at 4°C
- Remove supernatants for analysis of secreted mediators (TX-100 or CHAPS lysed represent total granule-derived proteins)

Secretion assay plate layout:

	1 Control #1 ↓	2 Control #2 ↓	3 Control #3 ↓	4	5 Inhibitor #1 ↓	6 Inhibitor #2 ↓	7 Inhibitor #3 ↓	8	9	10 Control (no Ab) ↓	11 Blank (pCa 8) ↓	12 0.5% CHAPS Lysed cells ↓
A	0.1 µg/ml →											
B	0.22 µg/ml →											
C	0.46 µg/ml →											
D	1 µg/ml →											
E	2.15 µg/ml →											
F	4.64 µg/ml →											
G	10 µg/ml →											
H	Empty →											

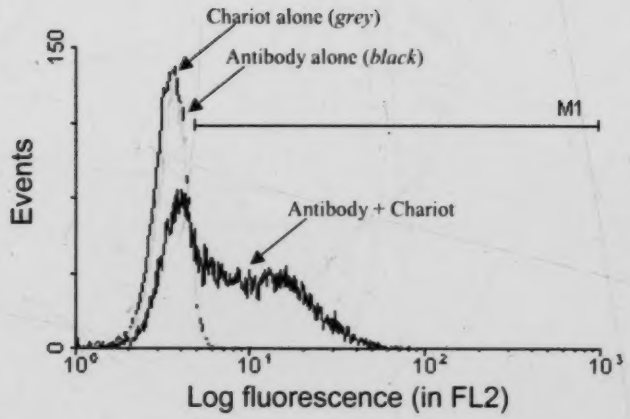

Figure B.1: Delivery of exogenous PE-labeled antibodies into eosinophils using Chariot protein conjugate. Eosinophils were isolated according to Chapter 2. Conjugation of PE-labeled goat anti-mouse phycoerythrin (PE) labeled antibodies (Cedarlane: see Chapter 2) to Chariot and incubations with cells were performed according to the manufacturer's specifications (Active Motif, Carlsbad, CA).Eosinophils were incubated with goat anti-mouse PE antibodies alone or Chariot protein linker alone as controls. Following incubation, eosinophils were washed three times in PBS and examined by FACS analysis for log fluorescence intensity. Shown is a representative of two similar experiments. The examination of two experiments revealed that approximately 60% of eosinophils exhibited a right shift in fluorescence intensity from controls (M1 gate shown).

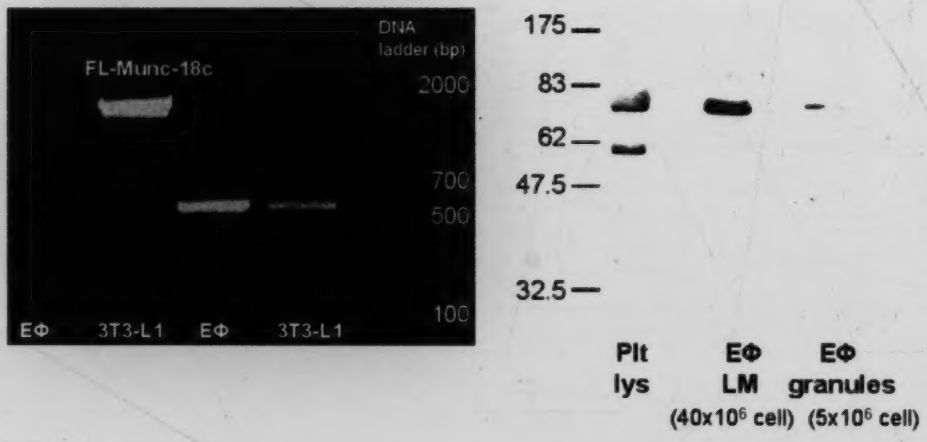

Figure B.2: Expression of munc18-c related isoform, platelet sec1 protein (PSP) in human eosinophils.

***(Left)* mRNA expression of munc-18c products obtained for human eosinophils.** Total RNA isolated from eosinophils was subjected to RT-PCR using full length primers to human munc18-c (*forward*: 5'-CTAAAGAGCGTCGTGTGGCAG -3', *reverse*: 5'- AGCACAACGCACTTCAGAGA -3') or primers designed to generate a truncated product of munc18-c (XXX bp) (*forward* : 5'-GCCACCTTGGATGAAAATCC-3', *reverse*: 5'-AGCAGGTCCTGTTCAGTTTTGC-3'). RT-PCR was performed as described in Chapter 2 using RNA from themurine adipocyte cell line, 3T3-L1 as a positive control. Products obtained using the full length primers (leftwo lanes) and truncated primers (right two lanes) are shown. The eosinophil product was subcloned and sequenced (n=1). Sequence analysis revealed that the eosinophil product was 100% homologous to the reported sequence for human PSP, a related isoform to munc18-c (Genbank accession # AF032922) (Reed. Et al. 1999. Blood 93(8) 2617-26).

***(Right)* PSP expression in eosinophil subcellular fractions.** Membrane-enriched pellets (40 X 10^6 cells) and EPO-enriched granule fractions (5 x 10^6 cells: ~ 50 µg protein) were prepared as described in Chapter 2 and subjected to SDS-PAGE. Platelet lysate (described in Chapter 2) was used as a positive control (50 µg). Western Blot analysis was carried out using rabbit antiserum antibodies generated against human PSP (1:1000) (a gift from G. Reed, Cadiovascular Biology Laboratory, Harvard School of Public Health, Boston, MA. An immunoreactive band of the predicted size (~70 kDa) is shown. An extra non-specific band of ~55-60 kDa is also evident for platelet lysates, consistent for this antibody. Molecular weight markers (kDa) are shown on the left.

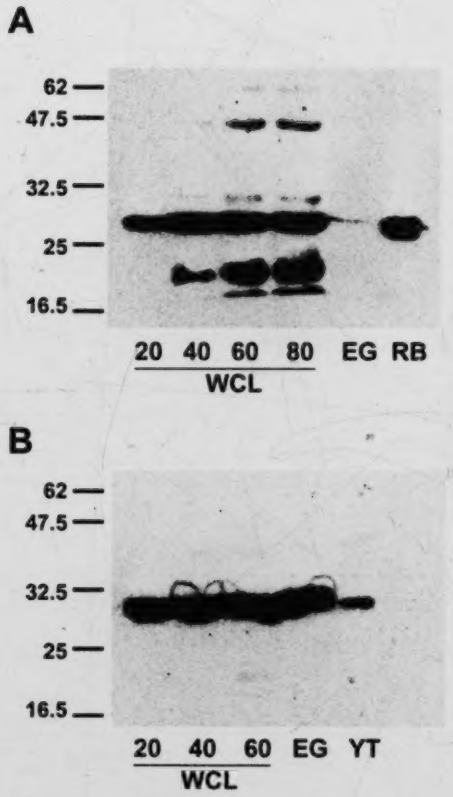

Figure B.3: Expression of Rab 4 and Rab27 in human eosinophils. Eosinophil whole cell lysates (WCL) (20-80 μg) and 10 μg EPO^2 granule fractions (EG) were subjected to SDS-PAGE and Western blotting with monoclonal antibodies to **(A)** Rab4 and **(B)** Rab27 (each at 1:1000) (purchased from BD biosciences: sodium azide free). Rat brain (RB) and Yt-Indy (YT) lysate positive controls are shown on the right for Rab4 and Rab27, respectively. (A) An immunoreactive band band of the predicted size for Rab4 (~30 kDa) was detected which co-migrated with the rat brain control. Additional immunoreactive bands were detected for WCL which may have reflected overloading of the gel or non-specific binding. The expression of Rab4 in EG was very low, suggesting it may not be a major component of crystalloid granule membranes. (B) A single band corresponding to Rab27 was detected which co-migrated with YT lysate. An immunoreactive band for Rab27 was apparent in EG, suggesting it may be involved in docking and/or tethering of crystalloid granules.
This data was generated by Michael Cheung (AHFMR summer student 2003) under the supervision of M. Logan